Full Title: **Dabigatran prevents lipopolysaccharide mediated apoptosis in zebrafish through a thrombin independent mechanism**

Short Title: **Dabigatran prevents lipopolysaccharide induced toxicity**

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Abstract: Endotoxemia is a feature of sepsis pathogenesis and has also been found to mediate the pathophysiology of multiple chronic inflammatory conditions. In this work, we use a lipopolysaccharide (LPS) induced endotoxemia model in zebrafish to identify novel mediators of LPS toxicity. We performed transcriptomic studies on LPS-treated larvae, followed by *in silico* analysis, which revealed associations between the signatures of LPS-treated embryos and those of drugs involving diverse pathways. In parallel, we performed an *in vivo* screen using >1,500 FDA-approved compounds and identified multiple novel small molecules that reduced inflammation and prevented LPS toxicity. We focused on the direct thrombin inhibitor dabigatran, which was identified through both the *in vivo* and *in silico* analyses. We found that dabigatran co-administration significantly reduced the expression of inflammatory cytokines and completely protected zebrafish from endotoxemic death due to LPS. Surprisingly, we found that this protection occurs in prothrombin mutant fish, proving that protection from endotoxemia occurs independently of the anticoagulant function of dabigatran. We additionally found that dabigatran administration significantly decreased nitric oxide production and apoptosis compared to LPS treatment alone, suggesting possible mechanisms by which protection from endotoxemia is achieved. In summary, we identify several novel small molecules that prevent LPS-induced endotoxemia and show that one such small molecule, dabigatran, exerts a thrombin-independent effect on nitric oxide production and apoptosis. This and the other identified small molecules warrant further exploration in inflammatory conditions including sepsis.

Author Summary: Sepsis is a common and deadly illness caused when there is an uncoordinated and overwhelming response to an infection. Endotoxemia is a feature of sepsis and occurs when part of the outer cell wall of bacteria called lipopolysaccharide (LPS) is released into the bloodstream, causing an excessive inflammatory response. To identify candidate drugs that could be repurposed to combat human disease, we screened through over 1500 FDA approved small molecules for protection against LPS induced toxicity using zebrafish as a model organism. In parallel, we undertook an *in silico* approach and compared the gene signature of LPS-treated embryos to the gene signature of small molecules published in the literature. We identified several novel drugs that could protect against LPS using these approaches. We further found that one such drug, the anticoagulant dabigatran, blocks LPS mediated cell death through a novel mechanism, independent of its primary target. Our work sets the stage for dabigatran and the other candidate drugs to be explored as therapeutics in human inflammatory conditions including sepsis.

61 **Introduction:**

62 Lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria, induces an immune
63 response upon recognition by Toll-Like Receptors (TLRs) (1, 2). LPS accounts for the relative
64 impermeability of the outer membrane of gram-negative bacteria to hydrophobic molecules,
65 making them resistant to many antimicrobials (3, 4). LPS-mediated endotoxemia mimics many
66 features of sepsis pathogenesis and might also play a causative role in the pathophysiology of
67 multiple chronic inflammatory disorders, including Parkinsons, Alzheimers, obesity, and
68 atherosclerosis (5). Previous work in mammals suggests that the translocation of gut-derived
69 LPS into the circulation might mediate some of these disease states (6, 7). Additionally, LPS
70 contributes to disease pathogenesis through its action as a prothrombotic molecule; LPS-induced
71 inflammatory mediators activate procoagulant pathways, induce platelet activation, downregulate
72 anticoagulant pathways and promote disseminated intravascular coagulation (8, 9). Moreover,
73 macrophages undergoing pyroptosis release tissue factor, further activating the coagulation
74 cascade (5, 9).

75 Given its role in multiple diseases, efforts have been made to block LPS biogenesis,
76 neutralize endotoxin, and hinder LPS-mediated immune activation (10-13), with many of these
77 attempts made in the context of sepsis. However, there is the possibility that therapeutics
78 targeting LPS signaling can also mitigate the development of chronic inflammatory conditions
79 such as those listed above.

80 In this work, we undertook large-scale *in silico* and *in vivo* analyses to identify novel
81 small molecule mediators of LPS toxicity. Murine and other mammalian models are cost-
82 prohibitive for the same types of large-scale *in vivo* screening studies. Zebrafish, on the other

83 hand, allow for a speed of discovery not possible with mammals as they combine the scalability
84 of *in vitro* systems with the complexity of a whole vertebrate organism.
85 In zebrafish, LPS-induced inflammation has been provoked either by injection into the
86 embryonic yolk sac or immersion of embryos and larvae into LPS-containing media (14-16).
87 Incubation with LPS-containing media allows uniform, rapid treatment of large numbers of fish.
88 A response remarkably similar to that in mammals is observed, including inflammation, platelet
89 activation, vascular leakage, and organ damage (16). Thus, many of the cardinal features of
90 sepsis pathogenesis are recapitulated by this model.
91 We performed novel high throughput studies in a zebrafish model of LPS-provoked
92 endotoxemia with the goal of identifying drugs that could be repurposed to combat acute and
93 chronic inflammatory conditions. We conducted an *in silico* analysis, where we compared RNA-
94 seq data from LPS-treated fish to publicly available RNA-seq datasets, revealing associations
95 between LPS and drugs involving disparate pathways. In parallel, an *in vivo* pathway analysis
96 was performed using >1,500 FDA-approved compounds to find those that mitigated LPS-
97 induced endotoxemia, uncovering multiple novel small molecules. We focused on the sole
98 anticoagulant (dabigatran) identified in both the *in silico* and *in vivo* analyses. Interestingly, the
99 administration of dabigatran reduced the upregulation of inflammatory cytokines, significantly
100 reduced nitric oxide production and apoptosis and abolished larval mortality from endotoxemia
101 due to LPS. Surprisingly, this protection was found to occur independent of its inhibitory effect
102 on thrombin. These data implicate dabigatran and other novel small molecules as anti-
103 inflammatory agents that can be explored as potential therapeutics in acute and chronic
104 conditions.
105

106 **Results:**

107 ***LPS incubation results in inflammatory and coagulopathic changes in zebrafish embryos***

108 *S. typhi* LPS was administered to zebrafish by incubation starting at 3 days post
109 fertilization (dpf) in accordance with published data (16). A dose-dependent effect on lethality
110 was noted over 24 hours. Hereafter, the lethal dose of LPS refers to the smallest amount that
111 caused uniform lethality by 4 dpf, which was slightly different (ranging from 40-50 μg/mL)
112 depending on the batch. A new dose-response curve was made for each batch of LPS tested, and
113 an example is shown in Fig. 1A. In addition to increased mortality, heart rate was significantly
114 decreased (Fig. 1B), and *grk5,* a gene involved in heart contractility, was significantly
115 downregulated 3 hours after LPS administration (Fig. 1C).

116

117 **Figure 1: LPS incubation results in dose-dependent lethality and signs of end-organ**
118 **damage in zebrafish larvae.** (A) Percent survival of 4 days post fertilization (dpf) zebrafish
119 larvae incubated with *S. typhi* LPS at 3 dpf. 3 clutches of 15 fish (total of 45) were used per
120 concentration. (B) Heart rate (beats per minute) was counted 3 hours after LPS (40 μg/mL)
121 administration and compared to controls. Each point represents a different fish, and the results
122 include 3 separate experiments. (C) *grk5*, a gene involved in heart rate contractility, was
123 measured by qPCR in LPS-treated fish compared to controls. Larvae from two or more clutches
124 were pooled, where each point represents a separate clutch, and the average fold change in LPS-
125 treated fish vs. controls was graphed. 30-45 total fish were used per time point. Bars indicate
126 mean and standard deviation. Student's t-test (two-tailed) on $\Delta\Delta C_t$ values was used to determine
127 significance. *P<0.05.

128

129 To determine the significant biological pathways altered upon LPS administration, RNA-
130 seq was performed on treated fish compared to controls as previously described (Guirgis et al.,
131 2023). Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis was performed
132 and revealed that "Pathogenic *E. coli* Infection" was the most significantly altered pathway in
133 LPS-treated fish (Fig. 2A, complete analysis is shown in Table S1), confirming that
134 administration induces a response akin to that provoked by bacteria. As expected, immune and
135 interferon pathways such as TNF signaling were significantly changed (Fig. 2A). A subset of
136 genes from these pathways were confirmed by qPCR and trended toward upregulation, with the
137 genes *il1b*, *il6* and *irg1l* significantly upregulated (Fig. 2B). In addition to provoking a
138 proinflammatory response, LPS is also known to be a prothrombotic molecule (7, 9). RNA-seq
139 data also showed that the complement, coagulation, and platelet activation pathways were
140 significantly altered in LPS-treated zebrafish compared to controls (Fig. 2A). A subset of
141 procoagulant genes from these pathways was upregulated, which was confirmed by qPCR (Fig.
142 2B). Interestingly, tissue factor b (gene *f3b*), but not tissue factor a (gene *f3a*) was significantly
143 upregulated in LPS-treated fish compared to controls, suggesting possible arterial injury as *f3b* is
144 thought to play a primary role in arterial, rather than venous, circulation (17). Time to occlusion
145 (TTO) after venous and arterial laser-mediated endothelial injury was measured as previously
146 described (17, 18) and is illustrated in Fig. 2D. The TTO was found to be significantly increased
147 in fish administered LPS for both systems (Fig. 2E, F). This change suggests a consumptive
148 coagulopathy phenotype similar to what we have observed with the loss of natural anticoagulants
149 (18, 19).
150

151 **Figure 2: The immune and interferon signaling pathways and procoagulant pathways are**
152 **significantly upregulated in LPS-treated fish compared to controls.** (A) KEGG pathway
153 analysis of RNA-seq data from whole LPS treated fish compared to controls. Significantly
154 changed genes (adjusted p-value <0.05) from RNA-seq data on pooled 3 dpf LPS-treated vs.
155 control fish (consisting of 30-45 embryos per category) performed in triplicate were used for
156 further analyses. These genes were converted to human versions and then submitted for KEGG
157 Pathway Analysis using the Enrichr platform, a publicly available tool developed by the
158 Ma'ayan lab at Icahn School of Mount Sinai, NY (Chen et al., 2013, Kuleshov et al., 2016).
159 Overlap score is the number of significantly changed genes over the total number of genes in the
160 pathway. Combined score considers the z-score (a value's relationship to the mean) in addition
161 to the adjusted p-value. The combined score is used to assess the significance of gene set
162 enrichment findings, with higher values being more significant. Adjusted p-value <0.05 is
163 considered significant. Immune and interferon-related and complement and coagulation
164 pathways are shown. For complete analysis, see Table S1. (B) A subset of genes in the immune
165 and interferon pathways were tested by qPCR, using 3 dpf larvae treated with 40 µg/mL LPS vs.
166 control conditions. Larvae from two or more clutches were pooled, where each point represents a
167 separate clutch, and the average fold change in LPS-treated fish vs. controls is graphed. Bars
168 represent mean and standard deviation. Student's t-test (two-tailed) on $\Delta\Delta C_t$ values was used to
169 determine significance. *P<0.05. (C) A subset of procoagulant genes was tested by qPCR, using
170 3 dpf larvae treated with 40 µg/mL LPS vs. control conditions. Larvae from two or more clutches
171 were pooled, where each point represents a separate clutch, and the average fold change in LPS-
172 treated fish vs. controls is graphed. Bars indicate mean and standard deviation. Student's t-test
173 (two-tailed) on $\Delta\Delta C_t$ values was used to determine significance. *P<0.05. A schematic of the

174 laser injury assay used to measure time to occlusion is shown in (D), where the time to formation
175 of an occlusive thrombus after laser pulses were delivered to the posterior cardinal vein or dorsal
176 aorta was measured. 120 seconds was the maximum time each larva was observed. Time to
177 occlusion (TTO) after laser injury in the venous (E) and arterial (F) circulation was measured in
178 3 dpf zebrafish larvae 3 hours after LPS administration and compared to controls. Each point
179 represents a separate fish, and the observer was blinded to the treatment condition. $P<0.05$ is
180 considered significant by Mann-Whitney *U* testing.
181
182 ***In silico and in vivo analysis reveals small molecule mediators of LPS-mediated endotoxemia***
183 To identify possible mediators of LPS toxicity, we initially undertook an *in silico* analysis
184 comparing the RNA-seq signature of LPS-treated embryos to those of genes and compounds
185 from publicly available databases (Table S2). After obtaining the list of small molecules related
186 to LPS based on the gene expression profiles (Table S2), we further looked into mechanisms of
187 action for each of these unique molecules as provided by the Connectivity Map (CMap)
188 database. Even though some compounds did not have an annotated MoA, the data showed an
189 intriguing pattern among the ones with detailed annotations. Notably, neuroactive compounds
190 such as dopamine and serotonin receptor antagonists, along with ion channel and kinase
191 inhibitors, emerged as the most commonly shared classes amongst the LPS-associated
192 compounds (Table 1).
193
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195
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Mechanism of Action (MoA)	How often MoA occurs among candidate drugs from Table S2
Dopamine receptor antagonist	10
EGFR inhibitor	9
Tubulin inhibitor	8
Adrenergic receptor antagonist	6
MEK inhibitor	6
PI3K inhibitor	6
Serotonin receptor antagonist	6
Calcium channel blocker	5
KIT inhibitor	5
Na/K-ATPase inhibitor	5
RAF inhibitor	5
JAK inhibitor	4
PDGFR inhibitor	4
PKC inhibitor	4
Topoisomerase inhibitor	4

Table 1: MoA of small molecules from Table S2 sorted by the number of times the MoA appears among the list of candidate drugs (only occurrences >= 4 are shown)

In parallel, we performed an *in vivo* analysis in zebrafish embryos. A lethal dose of LPS was co-administered at 3 dpf with an FDA-approved drug repurposing set consisting of >1500 compounds. Embryos were examined for survival 24 hours post-treatment, and each drug that was a 'hit' (i.e., enabled survival of at least one embryo) was validated in a larger cohort to confirm rescue. Approximately 20 small molecules were identified in this manner (Table 2). Most hits completely protected against LPS toxicity during the embryonic/early larval period, and others provided partial protection (Table 2, Fig. S1 A-T). These small molecule mediators of

211 LPS toxicity spanned multiple categories, most heavily represented by serotonin and dopamine
212 receptor modulators, chemotherapeutics, and anti-infectious agents. Similar to the *in silico*
213 analysis, small molecules modulating the dopamine and serotonin pathways and those affecting
214 tyrosine kinases were heavily represented *in vivo*. Four drugs were hits in both the *in silico* and
215 *in vivo* analyses; these were amsacrine, conivaptan, vilazodone and dabigatran etexilate (the
216 prodrug formulation of dabigatran, hereafter referred to simply as dabigatran) (Table S2, Table
217 2). Approximately half of the drugs identified *in vivo* (berberine, ketanserin, sunitinib, ceritinib,
218 fedratinib, nelfinavir, saquinavir, indacaterol, dabigatran) have been shown to ameliorate LPS
219 effects, and to function as mammalian anti-sepsis agents (20-40), suggesting conservation
220 between zebrafish and mammals in terms of the effects of these small molecules. The remaining
221 hits from the *in vivo* screen – ziprasidone, pergolide, vilazodone, bedaquiline, rucaparib,
222 pralsetinib, amsacrine, conivaptan, deserpidine, and lomitapide – are, to our knowledge, novel
223 mediators of LPS toxicity (Table 2). Despite some variability, there was an overall trend toward
224 decreased inflammatory cytokine production when a subset of these drugs were co-administered
225 with LPS and compared to LPS treatment alone (Fig. S2 A-L). Thus, these novel mediators of
226 LPS toxicity may function as anti-inflammatory agents.
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Drug Category	Drug identified from current zebrafish LPS screen	Main Mechanism of Action	Drug previously tested with LPS in mammals?/ Novel	Drug previously tested in sepsis?/ Novel
Dopamine and Serotonin Modulators	Ziprasidone	Dopamine and serotonin antagonism	Novel	Novel
	Pergolide	Dopamine receptor agonist	Novel	Novel
	Vilazodone	Inhibits serotonin transporter, partial agonist at serotonin receptors	Novel	Novel
	Brexpiprazole	Dopamine and serotonin receptor modulation	Novel	Novel
	Ketanserin	Serotonin receptor antagonist	Mouse (Liu et al., 2013, Sugino et al., 2009)	Clinical trial for septic shock (Vellinga et al., 2015)
Chemotherapeutics	Rucaparib	Inhibits poly(ADP-ribose) polymerase	Novel	Novel
	Sunitinib	Inhibits receptor tyrosine kinases	Mouse (Zhao et al., 2019)	Novel
	Fedratinib	Inhibits janus receptor kinase 2	Human macrophages (Akcora et al., 2019)	Novel
	Pralsetinib	Rearranged during transfection kinase inhibitor	Novel	Novel
	Ceritinib	Inhibits autophosphorylation of anaplastic lymphoma kinase	Human endothelial cells (Dayang et al., 2021, Hu et al., 2019)	CLP induced sepsis in mice (Hu et al., 2019)
	Amsacrine	DNA intercalator	Novel	Novel
Antiviral/ Antibacterial Agents	Nelfinavir	Protease inhibitor	Human macrophages (Wallet et al., 2012)	CLP induced sepsis in mice (Weaver et al., 2004)
	Saquinavir	Protease inhibitor	Mouse (Yao et al., 2023, Tong et al., 2021), Human lung cells (Zhang et al., 2018)	CLP induced sepsis in mice (Tong et al., 2021)
	Ledipasivir	Inhibits viral phosphorylation	Novel	Novel
	Bedaquiline	Blocks proton pump for ATP synthase	Novel	Novel

233

234 **Table 2: Small molecules protecting zebrafish embryos from LPS toxicity**

235

Drug Category	Drug identified from current zebrafish LPS screen	Main Mechanism of Action	Drug previously tested with LPS in mammals?/ Novel	Drug previously tested in sepsis?/ Novel
Miscellaneous	Indacaterol	β-adrenoreceptor agonist	Guinea pigs (Aritake et al., 2021), Human lung macrophages (Maarsingh et al., 2021)	Novel
	Conivaptan	Vasopressin receptor antagonist	Novel	Novel
	Deserpidine	Vesicular monoamine transporter blocker	Novel	Novel
	Lomitapide	Microsomal triglyceride transfer protein inhibitor	Novel	Novel
	Dabigatran	Thrombin inhibitor	Mouse (Kimura et al., 2020)	Mouse sepsis models, including CLP (Lei et al., 2021), *S. aureus* bacteremia in humans (Butt et al., 2021)
	Berberine	Activates AMPK (increases glycolysis)	Mouse (Chen et al., 2022), rats (Chen et al., 2021, Zhao et al., 2023), mouse macrophage cells (Zhao et al., 2023), bovine endometrial epithelial cells (Fu et al., 2023)	CLP-induced sepsis in mice (Chen et al., 2023), cecal slurry-induced sepsis (Li et al., 2021), *S. aureus*-induced septic arthritis in mice (Asila et al., 2022)

236 **Table 2: Small molecules protecting zebrafish embryos from LPS toxicity**

237

238 ***Dabigatran reduces LPS mediated inflammation and mortality***

239 Given the availability of tools to study coagulation in zebrafish (41, 42) we decided to

240 characterize further the one anticoagulant, dabigatran, that was identified through both *in silico*

241 and *in vivo* analyses. Although a prior study has shown only a modest effect of dabigatran on

242 lethality in sepsis models (31), we found that it completely averted LPS-induced lethality (Fig.

243 3A). Expression of inflammatory cytokines and procoagulant genes were downregulated in fish
244 co-treated with LPS and dabigatran compared to LPS alone (Fig. 3B). As previously shown (43),
245 time to venous occlusion was increased in dabigatran treated fish, indicating successful
246 anticoagulation (Fig. 3C). There was no additive effect on occlusion with co-treatment.
247
248 **Figure 3: The anticoagulant dabigatran mitigates LPS-induced lethality and reduces the**
249 **expression of inflammatory genes.** (A) Zebrafish were administered a lethal dose of LPS with
250 25 μM dabigatran along with appropriate controls and survival was tracked daily from 3 through
251 6 dpf. (B) Expression of inflammatory genes was evaluated by qPCR 3 hours after drug
252 administration in 3 dpf larvae treated with LPS and dabigatran. Pooled larvae (15-45) from a
253 clutch were used, where each point represents a separate clutch, and the average fold change in
254 LPS-treated fish vs. controls is graphed. Bars indicate mean and standard deviation. Student's t-
255 test (two-tailed) on $\Delta\Delta C_t$ values was used to determine significance. *$P<0.05$. (C) Time to
256 occlusion (TTO) after laser injury in the venous circulation was measured in 3 dpf zebrafish
257 larvae 3 hours after LPS and dabigatran administration. Each point represents a single larva, and
258 the observer was blinded to the condition. $P<0.05$ was considered significant by Mann-Whitney
259 *U* testing.
260
261 We next determined whether dabigatran administration could produce a more general
262 anti-inflammatory effect. Tail transection, an established method to induce inflammation, was
263 performed on 3 dpf larvae. We found that dabigatran did not reduce neutrophil or macrophage
264 recruitment to the tail transection site, suggesting that its protection might be more specific to
265 LPS rather than representing a general anti-inflammatory response (Fig. S3).

266 ***Dabigatran protects zebrafish from LPS toxicity independent of its anticoagulant function***

267 An anti-sepsis effect of dabigatran mediated through fibrinogen-like protein 2 (Fgl2) has been
268 described (31). To determine whether the protection from LPS toxicity was dependent on the
269 anticoagulant effect of dabigatran, we evaluated three other commonly used anticoagulants – two
270 affecting factor Xa, rivaroxaban and apixaban, as well as another thrombin inhibitor, argatroban.
271 Rivaroxaban and apixaban were previously shown to be active in zebrafish and were used here at
272 the same concentration (43). However, we required shorter incubation times due to the rapid
273 lethality induced by LPS. As expected, all anticoagulants increased the TTO (Fig. 4A, Fig. S4 A,
274 B). Interestingly, some of the anticoagulants appeared to have a synergistic effect with LPS,
275 significantly increasing the time to occlusion when compared to the drug alone (Fig. 4A, Fig. S4
276 A, B). However, none of these conferred protection from LPS toxicity (Fig. 4B, Fig. S4 C, D),
277 suggesting that anticoagulation might not be the mechanism through which dabigatran mitigates
278 LPS-induced endotoxemia.

279

280 **Figure 4: Dabigatran protection from LPS toxicity occurs independently of prothrombin.**
281 (A) Time to occlusion (TTO) after laser injury in the venous circulation was measured in 3 dpf
282 zebrafish larvae 3 hours after LPS and argatroban administration. Each point represents a single
283 larva, and the observer was blinded to the condition. $P<0.05$ was considered significant by
284 Mann-Whitney *U* testing. (B) Zebrafish were treated with argatroban and/or LPS at 3 dpf and
285 observed daily until 6 dpf for survival. (C) Three dpf larvae from *f2* heterozygous incrosses were
286 divided into four groups: untreated controls, 40 μg/mL LPS, 25 μM dabigatran, and
287 LPS/dabigatran. All groups were followed for 3 days for survival. (D) Genotype distribution of
288 surviving larvae.

289 To further test this hypothesis, we employed a prothrombin knockout background (null
290 mutation in the *f2* gene (43). Larvae from *f2* heterozygous incrosses were divided into four
291 groups: untreated controls, LPS, dabigatran, and LPS + dabigatran. Each group was followed for
292 survival over 3 days. LPS alone was lethal in all genotypes (Fig. 4C), revealing that thrombin
293 inhibition is not necessary for protection from LPS. The presence of homozygous mutants was
294 confirmed by genotyping all clutches, except for LPS alone due to their rapid degradation (Fig.
295 4D). Thus, dabigatran administration protected *f2* homozygous mutants from lethal LPS,
296 proving that its mechanism of protection occurs independently of thrombin inhibition.
297
298 ***Dabigatran administration reduces LPS mediated nitric oxide production and apoptosis***
299 Nitric oxide (NO) production can contribute to the hypotension seen in septic shock (44), and
300 LPS has previously been showed to increase NO (44). We confirmed that LPS administration
301 increased NO production in our system (Fig. 5A, B). Additionally, dabigatran has previously
302 been found to suppress nitric oxide synthase (45). We therefore tested whether dabigatran
303 treatment could reduce NO production and found this to be the case (Fig. 5A, B).
304
305 **Figure 5: Dabigatran reduces LPS mediated nitric oxide production.**
306 (A) Representative images of 3 dpf fish stained with DAF-FM DA to visualize nitric oxide
307 production after the indicated treatment. (B) ImageJ analysis showing mean fluorescence
308 intensity of images of 3 dpf DAF-FM DA stained fish with the indicated treatment; observers
309 were blinded to condition. Each point represents a different fish, and the results include 3
310 separate experiments. Student's t-test (two-tailed) on $\Delta\Delta C_t$ values was used to determine
311 significance. *P<0.05.

312 RNA sequencing data suggested that apoptosis related pathways were increased in LPS-
313 treated fish (Fig. 6A). As NO production has been shown to lead to apoptosis after LPS exposure
314 (46), we next tested whether this represented a mechanism by which protection from LPS is
315 conferred. We found that cell death (Fig. 6B, C) and apoptosis (Fig. 6D, E) were significantly
316 decreased in dabigatran and LPS co-treated fish. These data suggest that decreased NO
317 production and apoptosis might be mechanisms by which dabigatran protects against LPS
318 toxicity.
319
320 **Figure 6: Dabigatran reduces LPS mediated cell death and apoptosis.**
321 (A) KEGG pathway analysis of RNA-seq data from whole LPS treated fish compared to
322 controls. Significantly changed genes (adjusted p-value <0.05) from RNA-seq data on pooled 3
323 dpf LPS-treated vs. control fish (consisting of 30-45 embryos per category) performed in
324 triplicate were used for further analyses. These genes were converted to human versions and then
325 submitted for KEGG Pathway Analysis using the Enrichr platform, a publicly available tool
326 developed by the Ma'ayan lab at Icahn School of Mount Sinai, NY (Chen et al., 2013, Kuleshov
327 et al., 2016). Overlap score is the number of significantly changed genes over the total number
328 of genes in the pathway. Combined score considers the z-score (a value's relationship to the
329 mean) in addition to the adjusted p-value. The combined score is used to assess the significance
330 of gene set enrichment findings, with higher values being more significant. Adjusted p-value
331 <0.05 is considered significant. Apoptosis related pathways are shown. For complete analysis,
332 see Table S1. (B) Representative images of 3 dpf fish stained with acridine orange after the
333 indicated treatment. (C) ImageJ analysis showing mean fluorescence intensity of images of 3 dpf
334 acridine orange stained fish with the indicated treatment; observers were blinded to condition.

335 Each point represents a different fish, and the results include 3 separate experiments. Student's t-
336 test (two-tailed) on $\Delta\Delta C_t$ values was used to determine significance. *P<0.05. (D) Representative
337 images of 3 dpf fish stained with TUNEL after the indicated treatment. (E) Apoptosis was scored
338 in 3 dpf TUNEL stained fish with the indicated treatement; observers were blinded to condition.
339 Each point represents a different fish, and the results include at least 2 separate experiments.
340 Student's t-test (two-tailed) on $\Delta\Delta C_t$ values was used to determine significance. *P<0.05.
341

342 **Discussion:**

343 We have identified several small molecule mediators of LPS toxicity that can act as anti-
344 inflammatory agents. Approximately half had been previously explored in mammalian LPS
345 models, and all have been shown to reduce inflammation and ameliorate toxicity (20-23, 25-29,
346 32, 34, 35, 39, 40, 47-49), suggesting strong concordance between zebrafish and mammals.
347 These mediators were found to act through multiple mechanisms, including inhibition of TLR
348 and NFκB activation (25, 48) as well as inhibition of inflammation through NFκB-independent
349 mechanisms (26, 27). Other potential mechanisms include effects on apoptosis (22) and NOS
350 (nitric oxide synthase) activation (23). A smaller number of the identified drugs have also been
351 investigated in mammalian sepsis models (24, 31, 33, 35-37, 39, 50), all with beneficial effects,
352 indicating further concordance with our zebrafish data. The novel mediators of LPS toxicity that
353 have not previously been tested in other studies – amsacrine, bedaquiline, conivaptan,
354 deserpidine, lomitapide, pergolide, prasetinib, rucaparib, vilazodone, ziprasidone – have as yet
355 undetermined mechanisms of action and represent avenues for further research in both
356 mammalian inflammation and sepsis.

357 Clinical trials using anticoagulants in the context of sepsis have proven equivocal at best
358 (51-60), although it is still possible that specific subtypes of sepsis patients may benefit from
359 anticoagulant therapy. Previous studies have shown that dabigatran reduces inflammation ((31,
360 32) and can enhance survival in mouse models of sepsis (31), congruent with a cohort study
361 revealing that patients on dabigatran had a lower incidence of *Staphylococcus aureus* bacteremia
362 (33). Dabigatran has also been found to mitigate inflammation in multiple contexts, including
363 diet (61), oxysterols (62), and acute myocardial infarction (45). However, it is unclear whether
364 these beneficial effects were due to the anticoagulant function of dabigatran. Our data suggest
365 that dabigatran reduces inflammation and promotes survival from LPS toxicity through a novel
366 mechanism independent of its direct anticoagulant effect on thrombin.
367 We found that dabigatran administration reduces LPS mediated NO production and
368 apoptosis, suggesting that this is one possible mechanism by which dabigatran confers protection
369 from LPS. Dabigatran has previously been shown to suppress nitric oxide synthase in acute
370 myocardial infarction (45) and future experiments will clarify the mechanism by which
371 dabigatran reduces LPS mediated nitric oxide production.
372 There are several limitations of this study. Due to the stringency of the screen (mortality),
373 it is likely that other small molecules conferring beneficial effects but not affecting mortality
374 (e.g., those that reduce inflammation) were not identified. This study also does not necessarily
375 identify the mechanism by which each drug acts to reduce LPS toxicity. It is possible that the
376 effect is mediated through a non-primary or previously unknown mechanism of action (as is the
377 case with dabigatran).
378 Our data suggest that the novel small molecule mediators of LPS toxicity uncovered
379 through our screen can be explored as anti-inflammatory and anti-sepsis agents in mammals.

380 Given that the hits previously examined in mammalian systems were also found to have
381 beneficial effects in this zebrafish model, we expect that at least some of the novel drugs will
382 translate to mammalian models.
383 Previous work and our data suggest that dabigatran can act as an anti-inflammatory and
384 potential anti-sepsis agent (31-33). We show here definitive proof that dabigatran acts
385 independently of thrombin to exert its rescue of lethal endotoxemia. Venous thromboembolism
386 prophylaxis is the standard of care for most adult hospitalized inpatients, especially those
387 admitted for sepsis (63, 64). The most common agents used are unfractionated and low
388 molecular weight heparins, but the data from our study suggest the possibility that dabigatran
389 could be an alternative with a dual benefit –both anticoagulant and completely distinct anti-LPS
390 functions. Randomized controlled trials in human inflammatory conditions or inpatients would
391 be relatively easy to support given the successful use of direct oral anticoagulants and the
392 indication for anticoagulation in hospitalized patients (63, 64).
393
394 **Materials and Methods:**
395 ***Animal care and husbandry:***
396 Zebrafish (*Danio rerio*) were raised in accordance with animal care guidelines as approved by
397 the University of Michigan Animal Care and Use Committee. Wild-type fish were a hybrid line
398 generated by crossing AB and TL strain zebrafish acquired from the Zebrafish International
399 Resource Center. Genetic knockouts of prothrombin (*f2*) have been previously described (43).
400 Tricaine (Western Chemical) was used for anesthesia and rapid chilling in an ice water bath for
401 euthanasia.
402

403 ***Small molecule treatments:***

404 Small molecule screens (MedChemExpress FDA drug repurposing set) were started at 3 dpf,
405 where 3 larvae were plated per well in 96-well plates containing embryo media. All drugs were
406 initially tested at a concentration of 25 μM for resistance to LPS toxicity, and confirmatory
407 studies were performed with a larger group of fish (9-45) representing at least three different
408 clutches (3 separate experiments), where the drugs were tested at various concentrations.

409

410 ***Heart rate measurements:***

411 Heart rate was measured 3 hours after lethal LPS (40 μg/mL) administration and compared to
412 untreated controls. Larvae were mounted in 0.8% low melting point agarose and visualized using
413 an Olympus IX73 microscope. Heart rate was manually counted over 60 seconds for each larva.
414 Each experiment consisted of at least 15 larvae. At least three different clutches (3 different
415 experiments) were utilized.

416

417 ***Real-time quantitative polymerase chain reaction (RT-qPCR):***

418 RT-qPCR was performed as described previously (65). Total RNA was extracted from 3 dpf
419 whole larvae 3 hours post treatment using the RNeasy Mini Plus kit (Qiagen) and transcribed
420 into cDNA using Superscript III cDNA synthesis kit (Invitrogen). Transcript levels of genes of
421 interest were quantified by qPCR (Mastercycler realplex^2, Eppendorf) using Fast SYBR Green
422 Mix (Applied Biosystems, Waltham, MA). Three equal pools of whole larvae (15-45 embryos
423 per pool) were tested. Primer sequences are provided in Table S3. All expression data were
424 normalized to the *actb2* gene, and significance was analyzed using the double delta C_t method as
425 described (66).

426 ***RNA-seq preparation and analysis:***

427 RNA-seq was performed as described previously (65). Three biological replicate pools of 30-45
428 3 dpf whole embryos were used per condition. Total RNA was extracted, and cDNA was
429 prepared as above. Samples were sent to Novogene (Sacramento, CA) for further processing.
430 Paired-end 150 base pair (bp) sequencing was obtained with the Illumina NovaSeq 6000
431 Sequencing System. Reads were mapped to exonic regions based on the zebrafish reference
432 genome (GRCz11) using STAR36 (v.2.7.9a) and featureCounts37 (v.2.0.3). Differential
433 expression analysis was conducted using the R package DESeq2 ((67). All significant
434 differentially expressed genes (FDR adjusted p-values) were converted to human genes using the
435 R package babelgene. KEGG Pathway Analysis was then performed using the Enrichr platform,
436 a publicly available tool (68, 69). Overlap score is the number of significantly changed genes
437 over the total number of genes in the pathway. The combined score considers the z-score (a
438 value's relationship to the mean) in addition to the adjusted p-value; this score has been found to
439 outperform other ranking metrics (69). The combined score is used to assess the significance of
440 gene set enrichment findings, with higher values being more significant; values > 30 have been
441 considered significant in literature (18, 70).

442

443 ***Laser-mediated vascular endothelial injury:***

444 Larvae were treated with LPS and drug starting at 3 dpf and collected after 3 hours for
445 assessment of time to clot formation, performed as previously described (17, 18, 41, 43). Briefly,
446 3 dpf larvae were mounted in 0.8% low melting point agarose and visualized using an Olympus
447 IX73 microscope with an attached Micropoint focusing system and pulsed-dye laser (Andor).
448 For venous laser injury, 99 laser pulses were administered to the ventral surface of the posterior

449 cardinal vein, 5 somites caudal to the anal pore. For arterial laser injury, 99 laser pulses were
450 administered 5 somites caudal to the anal pore on the dorsal surface of the dorsal aorta. Time to
451 occlusion (formation of an occlusive thrombus) was measured, where 120 seconds was the
452 maximum time each larva was observed. Three biological replicates were performed for each
453 experiment.
454
455 ***In silico analysis:***
456 Differential gene expression data were retrieved from the RNAseq results as previously
457 described (65). The human orthologues of the respective genes were obtained from the Zebrafish
458 Information Network database (https://zfin.org/downloads/archive/2023.04.26) and the CLUE
459 software platform was utilized in performing Connectivity Map-based analyses
460 (https://www.sciencedirect.com/science/article/pii/S0092867417313090 (access date April 26,
461 2023)). Genes were ranked based on the adjusted p-values for the gene expression level changes,
462 and the top 150 upregulated or down-regulated genes were tested for the gene connectivity
463 evaluations across 64 human cell lines and disparate perturbation profiles. The connectivity data
464 were processed by using cmapR library in R (v4.2.2) environment (Natoli T (2023). cmapR:
465 Cmap Tools in R. R package version 1.12.0, https://github.com/cmap/cmapR). The data were
466 further filtered down to 27 cell lines of which most were hematopoietic/hepatic/pulmonary/non-
467 tumorigenic origins, or endothelial phenotypes (A375, A549, ASC, BJAB, CD34, HA1E,
468 HCC515, HEK293, HELA, HEPG2, HPTEC, HUH7, HUVEC, JURKAT, K562, MCF10A,
469 NCIH508, NCIH596, NOMO1, OCILY19, OCILY3, PHH, THP1, TMD8, U2OS, U937,
470 YAPC).
471

472 ***Acridine Orange Staining:***

473 Larvae were incubated in 10 µg/ml Acridine Orange stain (Sigma) for 15 minutes in embryo
474 media with LPS treatment and then rinsed three times before mounting in 0.8% agarose
475 and imaged with an Olympus IX73 microscope. Quantification of intensity was performed in
476 ImageJ.

477

478 ***TUNEL Staining:***

479 The TUNEL assay was performed according to the instructions provided in the In Situ Cell
480 Death Detection Kit, TMR red (Roche) for cryopreserved samples, with embryos cryopreserved
481 in Optimal Cutting Temperature Compound (OCT) and cryosectioned prior to labeling.
482 Images were quantified by an observer blinded to condition using a visual scoring system. The
483 visual scoring system consisted of grades 0 through 3 (0 = 0-9% fluorescence in visual field, 1 =
484 10-20% fluorescence, 2 = 30-70% fluorescence, 3 = 80-100% fluorescence).

485

486 ***NO Staining:***

487 Larvae were split into four treatment groups: control, LPS, dabigatran, and LPS with dabigatran.
488 Each treatment group was simultaneously incubated with 5 uM of DAF-FM-DA solution in the
489 dark at 25°C as previously described (Lepiller et al., 2007). After 2 hours larvae were washed
490 with embryo media and mounted in 0.8% agarose. Imaging was done using an Olympus IX73
491 microscope. Fluorescence quantification was obtained utilizing ImageJ.

492

493

494

495 ***Tail transection:***

496 Tg(lyzC:GFP)^{nz117} and TgBAC(mpeg1.1:EGFP)^{vcc7} transgenic larvae were anesthetized at 5 days
497 post fertilization and tail transection was performed with a sterile scalpel blade. Wounded larvae
498 were transferred into fresh embryo media with 25 µM dabigatran and incubated at 28.5 °C
499 for 5 hours prior to imaging using a Nikon SMZ25 stereoscope. Leukocyte recruitment was
500 quantified by measuring the fluorescent pixel area within 100 µm of the wound site in
501 FIJI/ImageJ.

502

503 ***Statistics:***

504 Statistics were performed using the GraphPad PRISM package. $P<0.05$ was considered
505 significant. For qPCR and RNA-seq data, Student's t-test on $\Delta\Delta C_t$ values was used to determine
506 significance. For time to occlusion (TTO) data, Mann-Whitney *U* testing was used to determine
507 significance.

508

509 ***Study Approval:***

510 Approval for zebrafish studies was granted by the University of Michigan Institutional Animal
511 Care and Use Committee, identification number PRO00010679 and the A*STAR Institutional
512 Animal Care and Use Committee, identification numbers 211667 and 221694.

513

514 **Acknowledgments:**

515 A*STAR IMCB Aquarium Platform for expert zebrafish husbandry.

516

517

518 **Competing Interests:**

519 J.A.S. has been a consultant for Sanofi, Takeda, Genentech, CSL Behring, Pfizer, Medexus and
520 NovoNordisk. The other authors declare that they have no competing interests to disclose.

521

522 **Funding:**

523 The authors were supported by the National Institutes of Health (https://www.nih.gov/)
524 [R35HL150784 (J.A.S.), K12HL133304 and K08GM151392 (V.J.) and the Charles Woodson
525 Collaborative Research Award (J.A.S and V.J.)], the Singapore National Medical Research
526 Council (https://www.nmrc.sg/)[OFIRG22jul-0081 (S.H.O.)]. J.A.S. is the Henry and Mala
527 Dorfman Family Professor of Pediatric Hematology/Oncology. X.S. holds the A*STAR
528 Singapore International Graduate Award (SINGA) scholarship (https://www.a-
529 star.edu.sg/Scholarships/for-graduate-studies/singapore-international-graduate-award-singa).

530

531 The funders had no role in study design, data collection and analysis, decision to publish, or
532 preparation of the manuscript.

533

534 **Data Availability:**

535 RNAseq data have been submitted to NCBI Gene Expression Omnibus and are available under
536 accession number GSE245299. Any other original data can be obtained by contacting the
537 corresponding author at vijacob@umich.edu.

538

539

540

Author Contributions:

V.J., J.A.S., S.O. and A.F. conceived and planned experiments. V.J., A.F., R.M., W.W. and X.S. performed and analyzed all experiments and M.Y. performed *in silico* analysis. V.J. wrote the manuscript, with input from all authors. J.A.S. revised the manuscript.

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785 **Figure S1: Multiple FDA-approved drugs protect zebrafish larvae from a lethal dose of**
786 **LPS.** (A)-(U) Zebrafish were administered a lethal dose of LPS along with compounds from an
787 FDA-approved repurposing library, and survival was tracked daily from 3 through 6 dpf. Any
788 experimental controls comprised the same solvent volume of each drug, either DMSO or water.
789
790 **Figure S2: Co-administration of identified drugs with LPS decreases inflammatory**
791 **cytokine expression.** (A)–(L) Expression of inflammatory genes was evaluated by qPCR 3
792 hours after drug administration in 3 dpf larvae treated with LPS, and each represented small
793 molecule. Pooled larvae (15-45) from a single clutch were used, where each point represents a
794 separate clutch, and the average fold change in LPS-treated fish vs. controls is graphed. At least
795 two biological replicates were performed. Bars indicate mean and +/- 1 standard deviation.
796 Student's t-test on $\Delta\Delta C_t$ values was used to determine significance. *$P<0.05$.
797
798 **Figure S3: Tail transection does not result in increased neutrophil or macrophage**
799 **migration to the site of injury.** 5-day-post-fertilisation (dpf) Tg(lyzC:GFP)^{nz117} and TgBAC
800 (mpeg1.1: EGFP)^{vcc7} transgenic embryos were anesthetized and tail transaction was performed
801 using a sterile surgical blade at the distal end of the notochord. After transection, embryos were
802 transferred into fresh E3 medium containing 25 μM dabigatran and kept at 28.5 °C. The
803 recruitment of neutrophils (A) and macrophages (B) to the wound site was imaged at 5 hours
804 post transection and quanitified by measuring fluorescence at the wound site.
805
806
807

808 **Figure S4: Other direct anticoagulants do not protect zebrafish larvae from LPS toxicity.**

809 (A),(B). Time to occlusion (TTO) after laser injury in the venous circulation was measured in 3

810 dpf zebrafish larvae 3 hours after LPS and anticoagulant administration. Each point represents a

811 single larva, and the observer was blinded to the condition. $P<0.05$ was considered significant by

812 Mann-Whitney *U* testing. (C), (D) Zebrafish were treated with apixaban or rivaroxaban and/or

813 LPS at 3 dpf and observed daily until 6 dpf for survival.

814

Figure 1

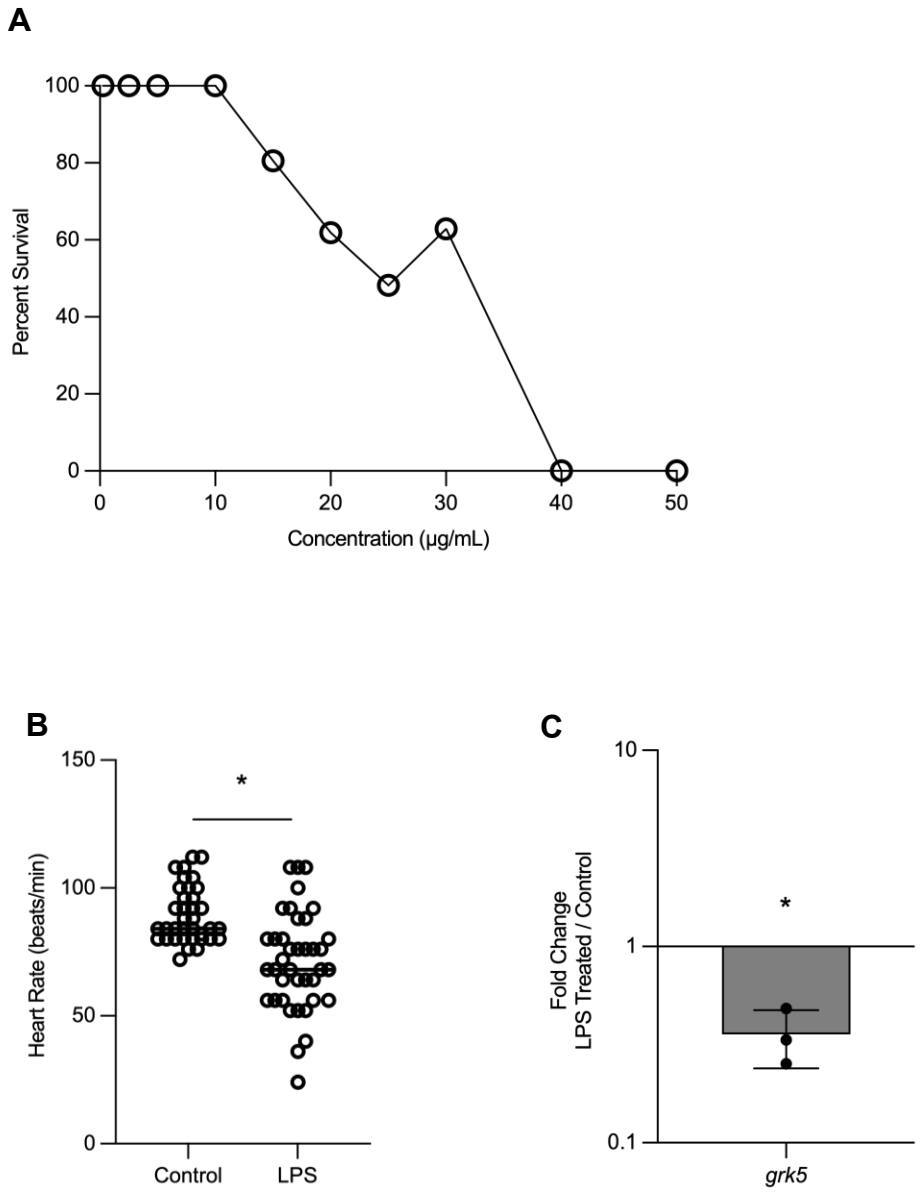
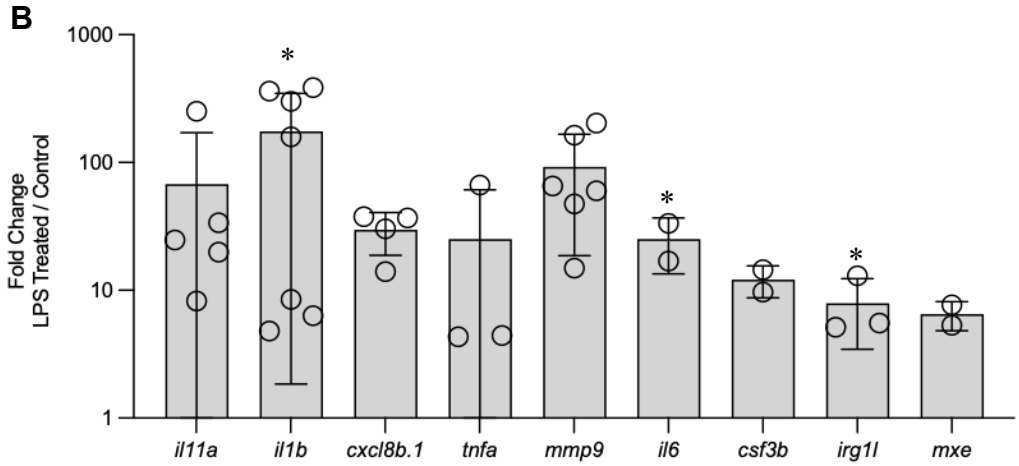
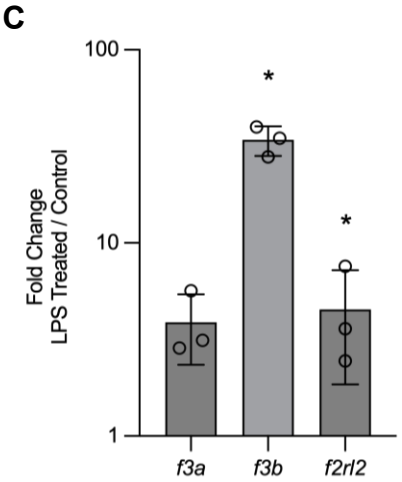

A
Percent Survival
100
80
60
40
20
0
0
10
20
30
40
50
Concentration (µg/mL)
B
Heart Rate (beats/min)
150
100
50
0
*
Control
LPS
C
Fold Change
LPS Treated / Control
10
1
0.1
*
grk5

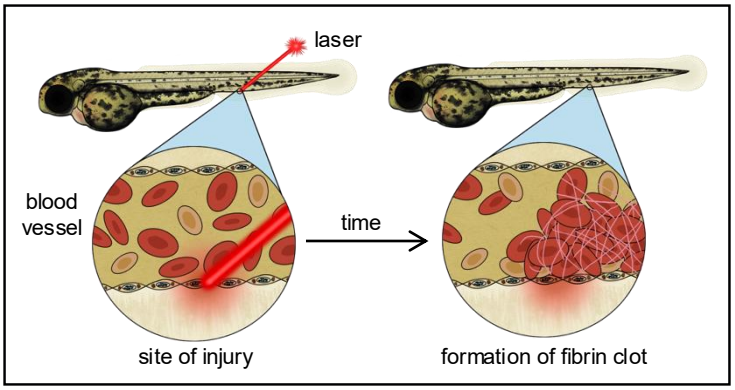
Figure 2

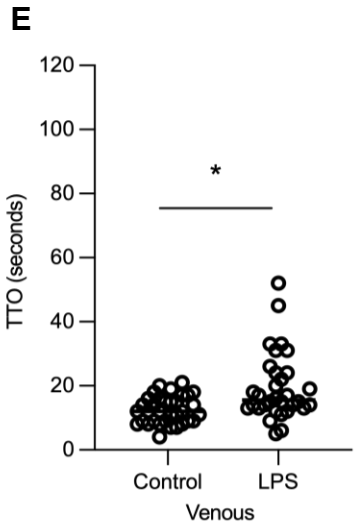
A

Name of Pathway	Overall Ranking (out of 98 significantly changed pathways)	Overlap Score	Adjusted P-value	Combined Score
Pathogenic *E.coli* Infection	1	38/197	2.59E-10	127.749924
Leukocyte Transendothelial Migration	9	21/114	8.13E-06	65.1873171
Complement and Coagulation Cascades	8	18/85	7.42E-06	78.4936729
TNF Signaling	12	20/112	1.84E-05	57.8681584
Platelet Activation	70	14/124	0.017	13.16661224









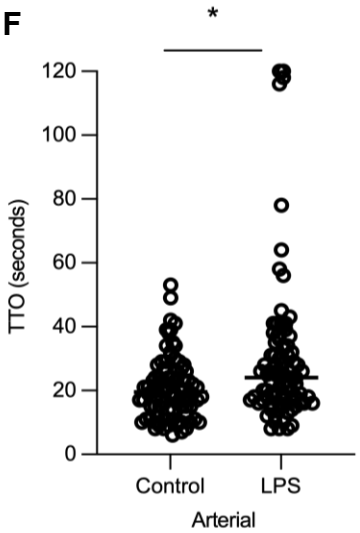


Figure 3

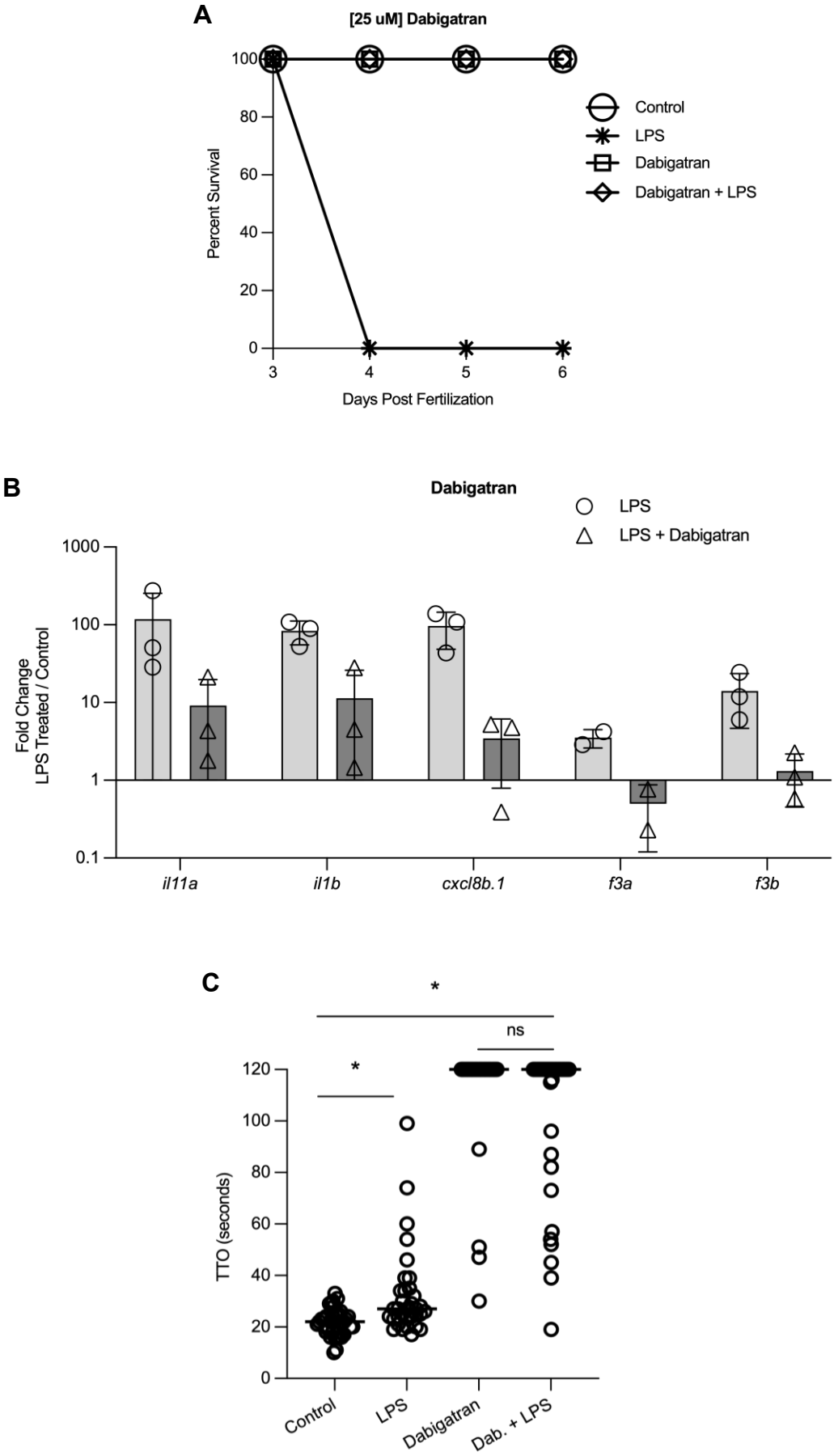

A
[25 uM] Dabigatran
Percent Survival
Days Post Fertilization
Control
LPS
Dabigatran
Dabigatran + LPS
B
Dabigatran
LPS
LPS + Dabigatran
Fold Change
LPS Treated / Control
il11a
il1b
cxcl8b.1
f3a
f3b
C
TTO (seconds)
ns
Control
LPS
Dabigatran
Dab. + LPS

Figure 4

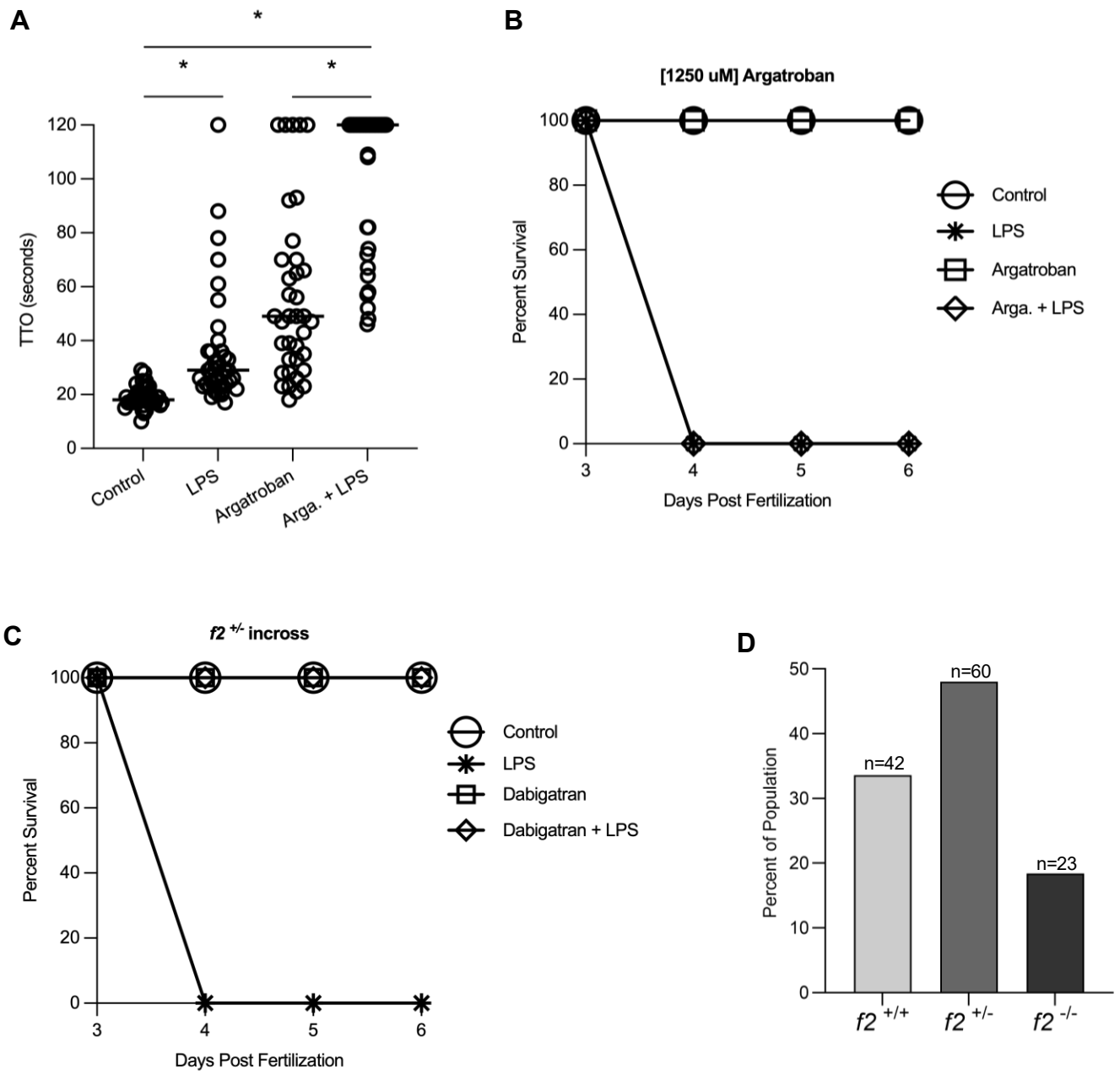
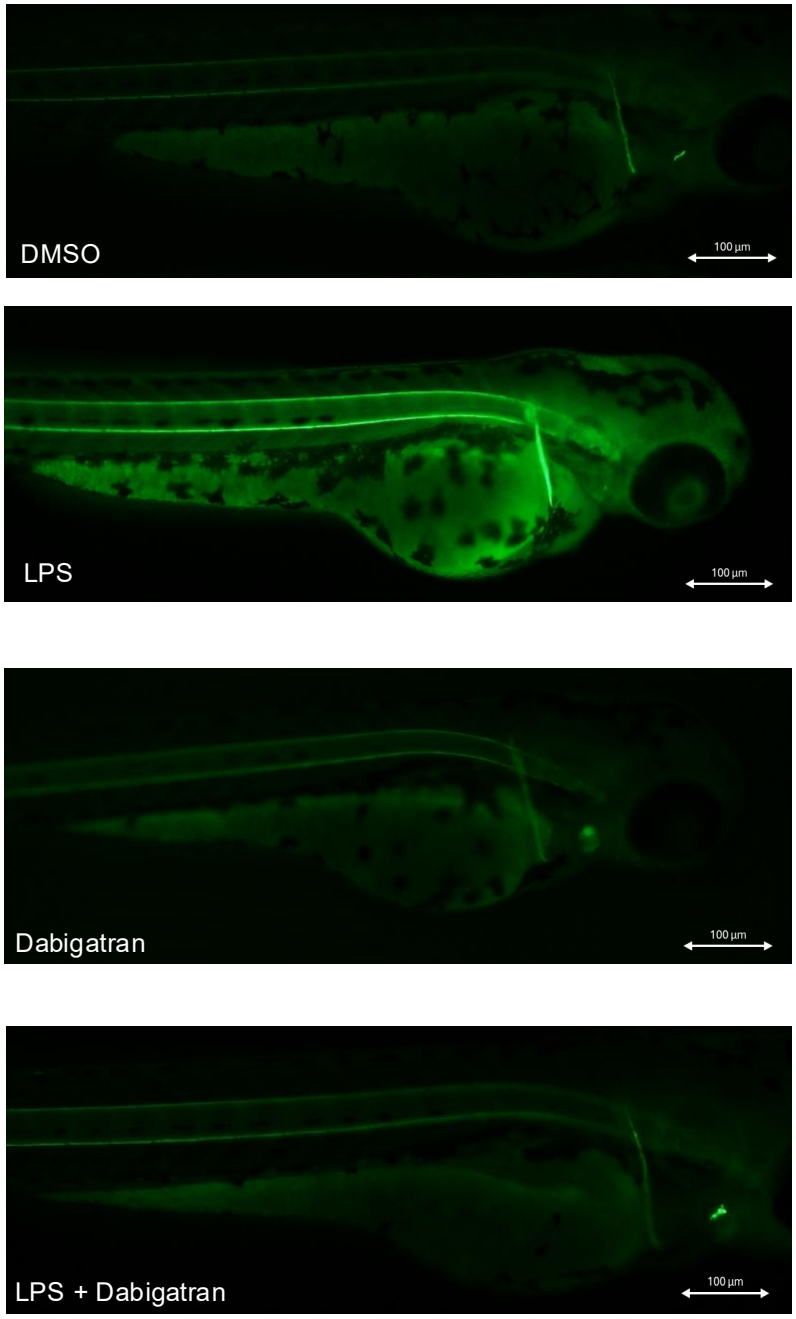

A
*
*
*
TTO (seconds)
120
100
80
60
40
20
0
Control
LPS
Argatroban
Arga. + LPS
B
[1250 uM] Argatroban
Percent Survival
100
80
60
40
20
0
3
4
5
6
Days Post Fertilization
Control
LPS
Argatroban
Arga. + LPS
C
f2 +/- incross
Percent Survival
100
80
60
40
20
0
3
4
5
6
Days Post Fertilization
Control
LPS
Dabigatran
Dabigatran + LPS
D
Percent of Population
50
40
30
20
10
0
n=42
n=60
n=23
f2 +/+
f2 +/-
f2 -/-

Figure 5

A


DMSO
100 µm
LPS
100 µm
Dabigatran
100 µm
LPS + Dabigatran
100 µm

B

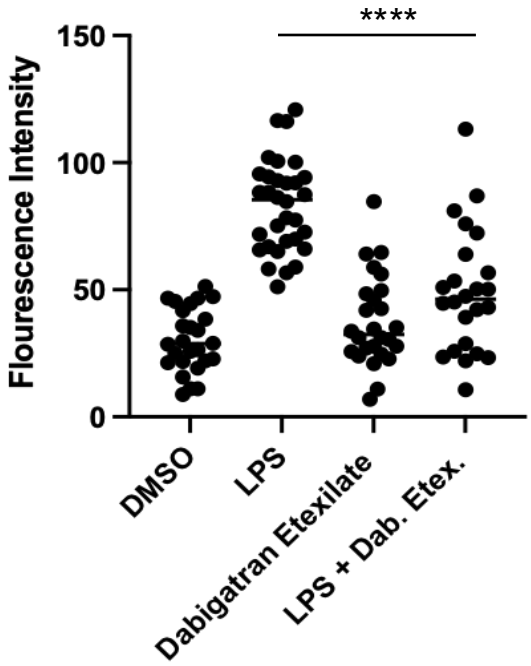
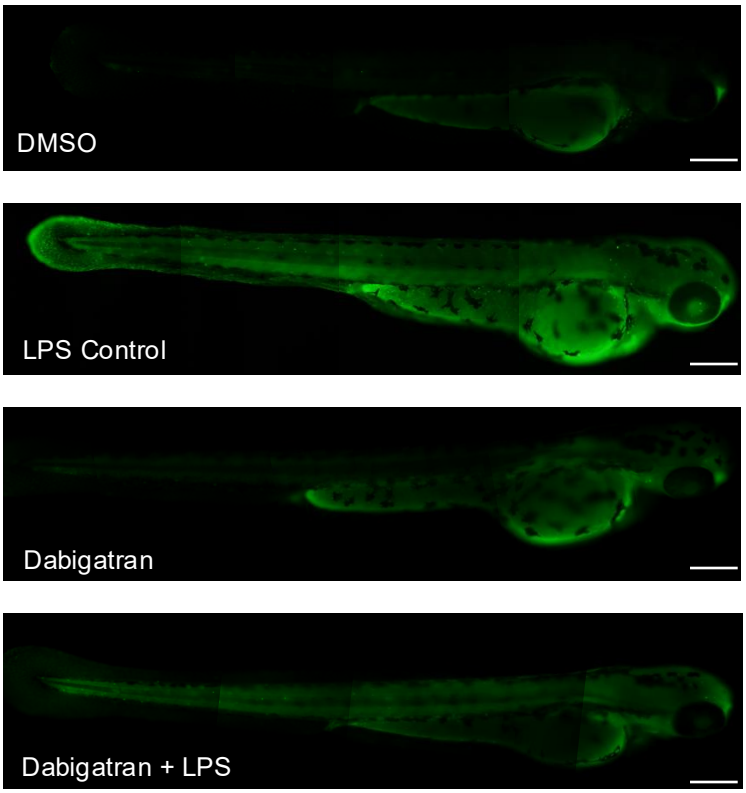

****
Flourescence Intensity
150
100
50
0
DMSO
LPS
Dabigatran Etexilate
LPS + Dab. Etex.

Figure 6

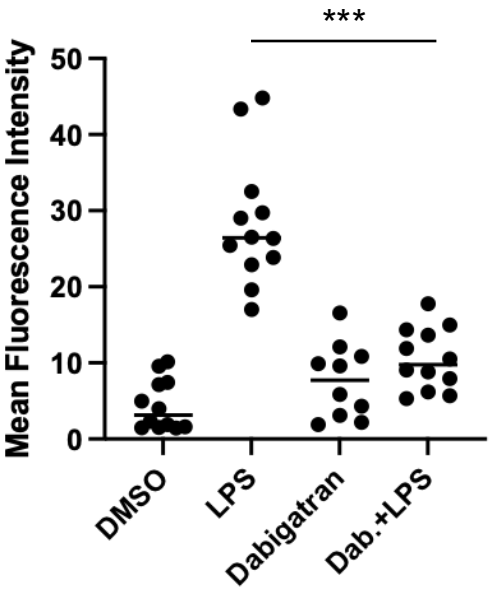
A

Name of Pathway	Overall Ranking (out of 98 significantly changed pathways)	Overlap Score	Adjusted P-value	Combined Score
Apoptosis	11	23/142	1.84 E-05	51.783249

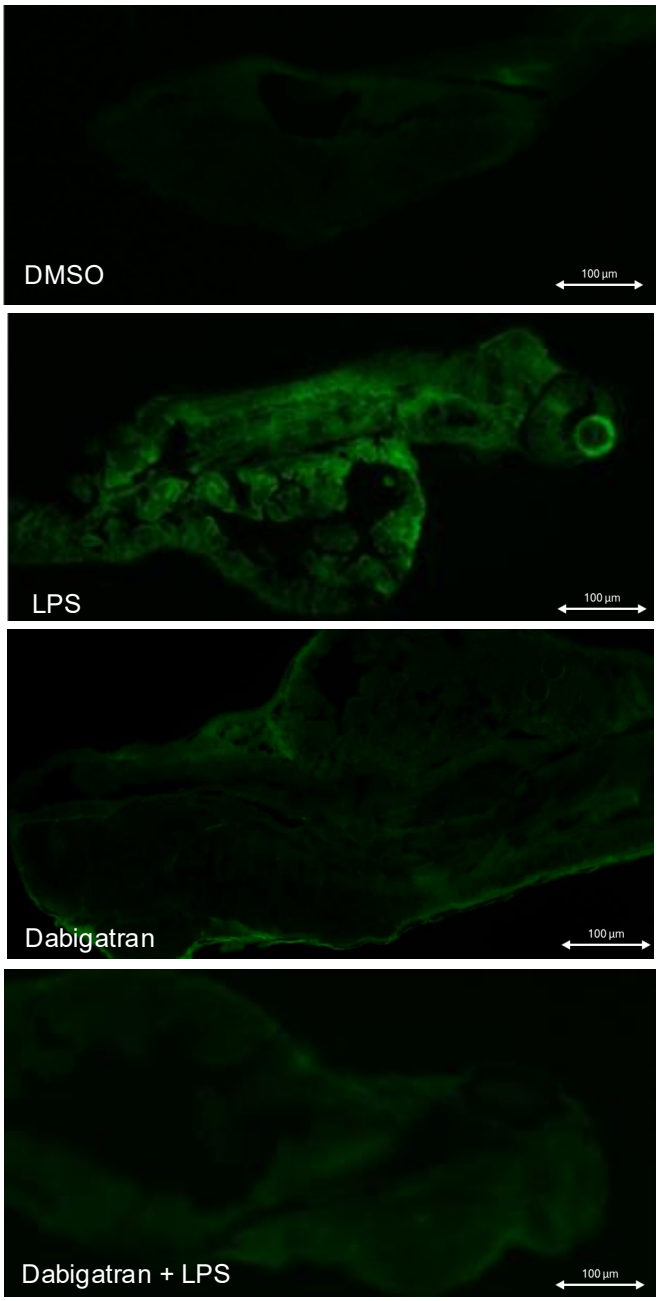
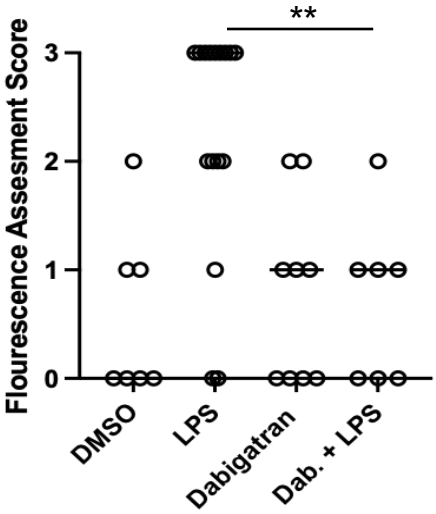
B



C



D



E