Inhibition of microbial deconjugation of micellar bile acids protects against intestinal permeability and liver injury

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Abstract

Altered host-microbe interactions and increased intestinal permeability have been implicated in the pathogenesis of a range of diseases. However, the mechanisms by which gut microbes affect epithelial barrier integrity remain unclear. Few host-produced metabolites that protect against epithelial damage have been identified, and whether microbial metabolism of host factors alters intestinal barrier function is unknown. Here, we investigate the effects of bacterial metabolism of host-produced bile acid (BA) metabolites on epithelial barrier integrity. We observe that rats fed a choline-deficient, high-fat diet (CDAHFD) exhibit reduced abundance of host-produced conjugated BAs in the intestine at early timepoints coinciding with increased permeability. We show that in vitro, conjugated BAs protect gut epithelial monolayers from damage caused by bacterially produced unconjugated BAs through micelle formation. We then demonstrate that inhibition of BA deconjugation using a small molecule inhibitor of gut bacterial bile salt hydrolase (BSH) enzymes prevents development of pathologic intestinal permeability and hepatic inflammation in CDAHFD-fed rats. Finally, we show that the predominant conjugated BAs in humans protect against epithelial barrier disruption in vitro. Our study identifies a protective role for conjugated BAs in intestinal epithelial barrier function and suggests that rational manipulation of microbial BA metabolism could be leveraged to regulate gut barrier integrity.

Introduction

In health, the intestinal epithelium forms a dynamic and tightly sealed barrier that is selectively permeable (Buckley & Turner, 2018). However, under pathologic conditions, tight junctions can become disrupted with excessive leakage of dietary and bacterial antigens, including lipopolysaccharide (LPS), into the portal and systemic circulation, directly inducing inflammation in extraintestinal organs (Massier, Bluher, Kovacs, & Chakaroun, 2021). Emerging data have implicated increased intestinal permeability in the pathogenesis of a range of human diseases, including inflammatory bowel disease (IBD), liver disease, type 1 and type 2 diabetes, cardiovascular disease, and depression (Bosi et al., 2006; Chopyk & Grakoui, 2020; Damms-Machado et al., 2017; Ohlsson et al., 2019; Pasini et al., 2016). The gut microbial communities of these patients are altered compared to healthy subjects, and gut microbial imbalance has been proposed to contribute to the development of intestinal permeability (Albillos, de Gottardi, & Rescigno, 2020; Chakaroun, Massier, & Kovacs, 2020). However, specific host and microbial factors that result in the development of pathologic intestinal permeability remain unclear.

Bile acids (BAs) have been implicated as potential causal agents in the development of pathogenic intestinal permeability (Murakami, Tanabe, & Suzuki, 2016; Papillon, Frey, Ford, & Gayer, 2013). BAs are steroidal natural products that are present in high concentrations in the gut (~200 μM to 1 mM in the large intestine) (Hamilton et al., 2007). In the liver, host enzymes convert cholesterol into conjugated primary bile acids, which contain a steroidal core appended to taurine or glycine through an amide bond linkage (**Figure 1A**). These compounds act as digestive surfactants; in humans, these metabolites are stored in the gallbladder and secreted into the small intestine post-prandially in order to aid in the absorption of lipids and vitamins (de Aguiar Vallim, Tarling, & Edwards, 2013). In the lower GI tract, bacteria convert primary conjugated bile acids into unconjugated primary BAs through the action of bile salt hydrolase (BSH) enzymes (Ridlon, Harris, Bhowmik, Kang, & Hylemon, 2016). BSHs are expressed in a

63 broad range of human gut bacteria (Song et al., 2019) and have no mammalian homolog (Foley,
64 O'Flaherty, Barrangou, & Theriot, 2019). Bacterial enzymes then further chemically modify
65 primary BAs, producing unconjugated secondary BAs. Following enterohepatic recirculation,
66 these molecules can be converted to conjugated primary and secondary BAs in the liver and then
67 re-secreted into the gut (Ridlon et al., 2016).
68
69 Previous work has demonstrated that exposure of epithelial monolayers to certain hydrophobic
70 BAs, including unconjugated BAs, leads to increased intestinal permeability in vitro and may
71 contribute to the development of intestinal inflammation and disruption of intestinal homeostasis
72 in vivo (Raimondi et al., 2008; Sarathy et al., 2017). In animal models, long-term administration
73 of a high-fat diet has been shown to lead to increased intestinal permeability associated with an
74 enrichment of hydrophobic BAs in the intestinal BA pool (Gupta et al., 2020; Stenman, Holma,
75 Eggert, & Korpela, 2013; Stenman, Holma, & Korpela, 2012). While unconjugated BAs have been
76 shown to increase epithelial permeability, the role of conjugated BAs in epithelial permeability is
77 unclear, and it is not known whether bacterial BA deconjugation affects intestinal homeostasis in
78 vivo.
79
80 Considering the close physiological relationship between the gut and the liver, optimal intestinal
81 barrier function is crucial for liver homeostasis (Nicoletti et al., 2019). Disruption of intestinal
82 barrier integrity may accelerate the pathogenesis of liver disease (Chopyk & Grakoui, 2020). Here,
83 we observe that increased intestinal permeability is an early feature of liver disease in rats fed a
84 choline-deficient, L-amino acid defined, high-fat diet, an animal model of liver cirrhosis
85 accompanied by pathogenic intestinal barrier damage (Longo et al., 2020). We also observed a
86 significant reduction in the abundance of intestinal and portal venous conjugated BAs in
87 CDAHFD-treated rats at early timepoints. Hypothesizing that conjugated bile acids could protect
88 the intestinal barrier against chemical insults, we demonstrate that tauro-conjugated BAs

89 sequester unconjugated BAs into micelles. We then show that this sequestration of unconjugated
90 bile acids protects epithelial cells from damage and permeability in vitro. Furthermore, using a
91 small molecule inhibitor of bacterial BSHs that we recently developed (Adhikari et al., 2021;
92 Adhikari et al., 2020), we show that inhibition of BSH activity increases conjugated BA abundance
93 in vivo and prevents the development of increased intestinal permeability, hepatic steatosis, and
94 hepatic inflammation in diseased rats. Finally, we demonstrate that glyco-conjugated BAs, the
95 predominant BAs in humans, also protect against epithelial damage caused by unconjugated BAs
96 in vitro. Together, these data indicate that gut microbial enzymes interact with host metabolites to
97 affect intestinal barrier function.
98
99 **Results**
100 **CDAHFD-fed rats develop increased intestinal permeability prior to developing hepatic**
101 **inflammation**
102 To investigate the molecular mechanisms leading to the development of intestinal permeability in
103 the context of an animal model relevant to human disease, we utilized a choline-deficient, L-amino
104 acid defined, high-fat diet (CDAHFD) in rats. CDAHFD is an established rodent model of diet-
105 induced cirrhosis that is characterized by disrupted epithelial barrier integrity (Zhong, Zhou, Xu,
106 & Gao, 2020). Previous studies have also found choline deficiency to be associated with
107 development of non-alcoholic fatty liver disease (NAFLD) and NASH in humans (Corbin & Zeisel,
108 2012; Guerrerio et al., 2012). CDAHFD-fed rats developed cirrhosis in 12 weeks (Matsumoto et
109 al., 2013) while controls fed a high-fat diet with equivalent fat by weight developed microvesicular
110 steatosis without inflammation or fibrosis (**Figure S1A**). CDAHFD-fed rats gained less weight than
111 controls and exhibited significantly increased markers of hepatocellular injury, hepatic
112 hydroxyproline levels, and mRNA expression of fibrosis-related and inflammatory genes at 12
113 weeks post-diet intervention (**Figure S1B-C,** refer **Table S1** for all qPCR primer sequences).
114

115 To assess intestinal permeability, we measured portal lipopolysaccharide (LPS) in CDAHFD-fed
116 rats at 48 hours, 1 week, 6 weeks, and 12 weeks (**Figure 1A,B**). Remarkably, portal LPS levels
117 were higher in CDAHFD-fed rats compared to controls as early as 48 hours post-diet intervention
118 (**Figure 1C**), when markers of liver injury were not yet significantly elevated (**Figure S1C-E**).
119 Further, intestinal epithelium from CDAHFD-fed rats exhibited significantly increased
120 inflammation and epithelial hyperplasia at early time points (**Figure S1F**). These data suggest
121 that intestinal injury and permeability precede hepatic phenotypes in the CDAHFD model of liver
122 disease. To confirm these results, using a FITC-Dextran assay, we found that CDAHFD-fed rats
123 exhibited a significant increase in intestinal permeability compared to controls at 48 hours after
124 diet initiation (**Figure 1D**). We observed significantly increased membrane localization of ZO-1 in
125 intestinal epithelial cells of CDAHFD-fed rats at 48 hours that persisted after 1 week of diet (**Figure**
126 **1E**, **F**). Increased expression and plasma membrane localization of ZO-1 are indicative of
127 increased intestinal permeability (Guo, Wang, Liu, & Ye, 2018; Karczewski et al., 2010).
128 Moreover, treatment of gut epithelial cells with permeability agents results in dynamic changes in
129 ZO-1 subcellular localization, including recruitment to the plasma membrane (Guan et al., 2011;
130 Odenwald et al., 2017). As such, ZO-1 redistribution in the intestinal epithelium of CDAHFD-fed
131 rats is consistent with increased intestinal permeability. Importantly, at 48 hours, CDAHFD-fed
132 rats did not exhibit any evidence of hepatic inflammation compared to controls while inflammation
133 was apparent at 1 week post-dietary intervention (**Figure S1C-E**). Together, these results
134 demonstrate that intestinal permeability is an early feature of this animal model and precedes the
135 development of hepatic inflammation.
136

137 **Conjugated BAs are reduced in the cecum and portal veins of CDAHFD-fed rats at early**
138 **timepoints**

139 To assess whether changes in BAs are associated with increased intestinal permeability, we
140 performed intestinal and portal venous BA profiling in CDAHFD-fed and control rats using ultra-

141 high performance liquid chromatography-mass spectrometry (UPLC-MS). We observed a
142 significant decrease in the abundance of cecal and portal venous conjugated BAs in CDAHFD-
143 fed rats at 48 hours and 1 week after dietary intervention (**Figure 1G-H**). In particular, levels of
144 tauro-cholic acid (TCA) and tauro-alpha- and tauro-beta-muricholic acid (Tα/βMCA) were
145 decreased in both cecal contents and portal veins at both timepoints (**Figure S2-5)**. Total cecal
146 and portal venous BA concentrations were similar between the two groups at these early
147 timepoints (**Figure S2-5**). In addition, levels of the conjugated bile acids tauro-chenodeoxycholic
148 acid (TCDCA), tauro-deoxycholic acid (TDCA), and tauro-ursodeoxycholic acid (TUDCA) were
149 decreased and levels of the unconjugated BAs cholic acid (CA), chenodeoxycholic acid (CDCA),
150 α-muricholic acid (αMCA), and β-muricholic acid (βMCA) were increased in portal veins at the 1
151 week timepoint (**Figure S5**). These findings indicate that changes in cecal and portal venous BA
152 composition are associated with the onset of increased intestinal permeability in CDAHFD-fed
153 rats and suggest that these metabolites could play a causal role in development of gut barrier
154 damage.
155
156 Intestinal permeability and inflammation are regulated in part by the BA-sensing farnesoid X
157 receptor (FXR), which has been linked to NAFLD/NASH pathogenesis (Arab, Karpen, Dawson,
158 Arrese, & Trauner, 2017; Li et al., 2013). In addition, FXR signaling modulates a tight negative
159 feedback loop that controls BA synthesis in the liver (Fiorucci & Distrutti, 2015). At 48 hours after
160 dietary intervention, ileal *Fgf15* expression was similar in both groups when multiple lines of
161 evidence for increased intestinal permeability were already observed in CDAHFD-fed animals
162 (**Figure S6A)**. Expression of BA synthesis and conjugation enzymes were not significantly
163 affected in CDAHFD-fed rats at 48 hours of diet **(Figure S6B)**. Expression levels of BA transporter
164 proteins were also unaffected in CDAHFD-fed rats compared to controls **(Figure S6C)**. We found
165 that cecal BSH activity in CDAHFD rats was significantly increased at 48 hours compared to
166 control rats (**Figure S6D**) (Adhikari et al., 2020). These data suggest that the decrease in

167 conjugated BAs in CDAHFD-fed rats was driven at least in part by an increase in BA
168 deconjugation by gut microbes. Together, our findings suggest that FXR-independent
169 mechanisms are responsible for the earliest events that establish intestinal barrier dysfunction in
170 this animal model of pathogenic intestinal permeability.
171

172 **In vivo BA compositions reflect increased intestinal permeability as disease progresses**

173 UPLC-MS analysis revealed significant decreases in cecal unconjugated BAs in CDAHFD-fed
174 rats at 6 weeks (e.g., αMCA, LCA, DCA, UDCA) and in unconjugated, conjugated, and total BAs
175 at 12 weeks (e.g., CA, αMCA, βMCA, LCA, DCA, UDCA, TDCA, tauro-omega-muricholic acid
176 (TωMCA), TUDCA) compared to control animals (**Figure S7,8)**. Interestingly, at the same
177 timepoints, portal venous BA profiling revealed the inverse finding, with significantly higher
178 concentrations of total and unconjugated BAs (e.g., CA, CDCA, αMCA, βMCA) in CDAHFD-fed
179 rats compared to controls (**Figure S9,10)**. No significant differences were observed in the
180 expression of genes involved in BA synthesis (*Cyp7a1, Cyp8b1, Cyp27a1*) or transport (*Asbt*,
181 *Osta/Ostb*) (**Figure S11A,B**), indicating that the observed changes are unlikely to be the result of
182 decreased BA synthesis or increased BA transport. These findings are consistent with the
183 observation that CDAHFD-treated rats exhibit epithelial barrier damage and suggest that as
184 disease progresses, increased intestinal permeability results in leakage of intestinal BAs into
185 portal circulation.
186

187 **Conjugated BAs protect intestinal epithelial monolayers from unconjugated BA-mediated**
188 **damage in vitro**

189 Based on our in vivo observations, we hypothesized that groups of conjugated or unconjugated
190 BAs could be causally contributing to epithelial layer damage. To test this hypothesis, we utilized
191 Caco-2 cells in transwell inserts as an in vitro model system. Once differentiated, these cells form
192 a polarized monolayer that mimics the intestinal epithelium, and this system has been previously

193 used to study the effect of metabolites on gut layer integrity (Scott, Fu, & Chang, 2020). Gut
194 permeability was assayed by measuring FITC-Dextran (4 kDa) permeability through the
195 monolayer and quantifying fluorescence in the basolateral chamber (Chaudhari, Harris, et al.,
196 2021). We found that cecal extracts isolated from CDAHFD-fed rats at 48 hours and 1 week
197 induced increased permeability compared to cecal extracts from control rats (**Figure 2A**). These
198 findings demonstrate that intestinal contents from CDAHFD-fed rats induce epithelial barrier
199 permeability at early timepoints.
200
201 To test whether cecal BAs specifically induce epithelial permeability, we generated reconstituted
202 pools of BAs that mimic the average physiological concentrations observed in rat ceca and tested
203 their ability to induce permeability in Caco-2 monolayers in vitro. The 48 hour CDAHFD total BA
204 pool induced significantly higher permeability than the DMSO control (**Figure 2B**). Interestingly,
205 while the unconjugated BA pool alone induced a significant increase in permeability, the
206 conjugated BA pool did not damage the monolayer barrier integrity. Remarkably, at later time
207 points (1, 6, 12 weeks), the addition of conjugated BAs to unconjugated BAs mitigated the severity
208 of epithelial permeability compared to unconjugated BAs alone (**Figure 2B**).
209
210 We next hypothesized that cecal conjugated BAs protect against the disruption of the epithelial
211 integrity. To test this hypothesis, we generated equimolar pools of the predominant cecal BAs
212 that we detected in vivo. We treated differentiated Caco-2 monolayers with increasing
213 concentrations of either (1) unconjugated BAs (βMCA, CA, DCA, UDCA, CDCA); (2) conjugated
214 BAs (TβMCA, TCA, TDCA, TUDCA, TCDCA) or (3) combined BA pools, followed by the
215 permeability assessment (**Figure 2C**).
216
217 At physiological concentrations of BAs in CDAHFD-fed rat cecal contents (~1000-4000 μM),
218 epithelial monolayer integrity was compromised after addition of unconjugated BAs but damage

219 was prevented by addition of an equimolar concentration of conjugated BAs (**Figure 2D,**
220 **S2,3,7,8**). Conjugated BAs alone did not disrupt monolayer integrity at any concentrations tested.
221 Similarly, while unconjugated BAs were toxic to cells, equimolar addition of conjugated BAs
222 abrogated this effect (**Figure 2E**). Visualization of Caco-2 monolayers by hematoxylin and eosin
223 staining further confirmed the toxic effects of unconjugated BAs were largely rescued by addition
224 of conjugated BAs (**Figure 2F**). Finally, to assess the integrity of tight junctions in the epithelial
225 monolayers, we performed transmission electron microscopy (TEM) on Caco-2 epithelial
226 monolayers exposed to the above BA groups. We identified dilatations in the tight junctions of
227 Caco-2 cells exposed to unconjugated BAs alone not present in cells exposed to conjugated BAs
228 alone or to a combination of both (**Figure 2G, Figure S12**). These tight junction dilatations have
229 been observed in ileal enterocytes of Crohn's disease patients and correlate with increased
230 permeability (Soderholm et al., 2002). Together, these results demonstrate that while
231 unconjugated BAs damage epithelial layers, conjugated BAs protect against intestinal epithelial
232 damage and permeability in vitro.
233
234 **Conjugated BAs sequester unconjugated BAs through the formation of micelles**
235 We next sought to determine the mechanism by which conjugated BAs protect intestinal epithelial
236 monolayers from unconjugated BA-mediated permeability in vitro. BAs are detergents and
237 effectively solubilize fats and vitamins by forming micelles in the intestine (Hofmann, 1963). We
238 hypothesized that when combined, conjugated BAs form micelles with unconjugated BAs,
239 sequestering unconjugated BAs away from the epithelial cells. To test this hypothesis, we
240 assessed the critical micelle concentration (CMC) of these BA populations using a fluorescent
241 probe (Fluksman & Benny, 2019). Unconjugated BAs are expected to have a higher CMC
242 compared to conjugated BAs (Pavlovic et al., 2018). If micelles formed after combining
243 unconjugated and conjugated BA populations, we would expect the CMC of the combined
244 populations to be lower than that seen with unconjugated BAs alone. Consistent with this

245 hypothesis, the combined pool CMC was 4.2 mM while the unconjugated and conjugated BA pool
246 CMCs were 6.7 mM and 4.0 mM, respectively (**Figure 3A**). The addition of 80 mM urea prevented
247 micelle formation as previously described (**Figure 3A**) (Hofmann, 1963). We also performed direct
248 visualization of micelles using negative staining-electron microscopy. At 5 mM concentration, no
249 micelles were visualized in the unconjugated BA pool while micelles were seen in the conjugated
250 BA pool, consistent with our CMC determinations and total cecal BA quantification in CDAHFD-
251 fed rats at the 48 hour time point (**Figure 3B, S2**). Combining BA pools resulted in larger micelles,
252 while urea prevented micelle formation entirely. Thus, in the presence of conjugated BAs,
253 unconjugated BAs are sequestered in micelles.
254
255 To investigate whether the protective effects of conjugated BAs are micelle-dependent, we tested
256 whether BA pools induce intestinal permeability in the presence of 80 mM urea, a concentration
257 that prevents micelle formation (**Figure 3A-B**) without inducing toxicity in Caco-2 cells (**Figure**
258 **3C**). Notably, addition of urea resulted in loss of conjugated BA-mediated protection of epithelial
259 barrier integrity and cell viability (**Figure 3D-F**). We also exposed Caco-2 monolayers to combined
260 BA pools in the presence or absence of urea and quantified the amount of unconjugated BAs that
261 passed through the monolayer into the basolateral chamber by UPLC-MS. We observed
262 significantly increased amounts of unconjugated BAs in the basolateral chamber in the presence
263 of urea, providing further evidence that micelle formation prevents epithelial damage and
264 permeability (**Figure 3G**). Together, our data provides evidence that unconjugated BAs lead to
265 increased permeability across an intestinal epithelial monolayer by inducing cytotoxic effects.
266 Further, these effects are prevented by the addition of conjugated BAs, which form BA micelles
267 with unconjugated BAs in vitro (**Figure 3H**).
268
269 **BSH inhibition by AAA-10 prevents altered intestinal permeability and hepatic**
270 **inflammation in CDAHFD-fed rats**

271 Because conjugated BAs appear to protect against intestinal epithelial damage, we hypothesized
272 that a decrease in microbial BA deconjugation by BSH might attenuate intestinal permeability in
273 CDAHFD-fed rats. We recently reported the development of a covalent, gut-restricted, small
274 molecule inhibitor of gut bacterial BSHs, AAA-10, a compound that effectively inhibits BSH activity
275 and increases the abundance of conjugated BAs in vivo (**Figure 4A)** (Adhikari et al., 2021). We
276 thus hypothesized that treatment of CDAHFD-fed rats with AAA-10 would prevent increased
277 intestinal permeability. A dose of 10 mg/kg AAA-10 or vehicle control was administered via
278 gavage twice a day for 7 days to CDAHFD-fed rats (**Figure 4B)**. AAA-10 administration led to
279 ~20-30 μM AAA-10 in cecal contents 48 hours and 1 week post-gavage (**Figure 4C**). No AAA-10
280 was detected in peripheral blood (**Figure S13A**), a finding that is consistent with our previous
281 results (Adhikari et al., 2021) and indicates that this BSH inhibitor exhibits low systemic exposure.
282 We found that AAA-10 reduced cecal BSH activity of CDAHFD-fed rats (**Figure 4D**) and
283 significantly increased both the abundance of conjugated BAs as a group (**Figure 4E**) and the
284 concentrations of individual conjugated BAs (**Figure S14**) by 1 week. Notably, we observed
285 significant decreases in levels of portal venous LPS suggestive of increased intestinal barrier
286 function in AAA-10-treated rats compared to vehicle-treated animals (**Figure 4F**). Furthermore,
287 we observed normalization of ZO-1 localization and expression, suggesting that AAA-10
288 treatment prevented the development of intestinal permeability in CDAHFD-fed rats (**Figure 4G**).
289

290 As intestinal permeability has been linked to translocation of intestinal products that further
291 exacerbate hepatic inflammation (Chopyk & Grakoui, 2020), we evaluated the impact of 8 day
292 AAA-10 treatment on liver phenotypes (**Figure 5A**). Consistent with our previous results, we found
293 that the BA pool shifted toward conjugated BAs in AAA-10-treated compared to control-treated
294 rats (**Figure S15**). Mild weight loss was observed in the AAA-10-treated animals (**Figure S16A**),
295 consistent with our previous report that genetic removal of bacterial BSH causes altered metabolic
296 phenotypes including reduced weight gain on a high fat diet (Yao et al., 2018). We did not observe

297 significant weight loss in AAA-10-treated rats in the 7 day experiment (**Figure S13B**), and we
298 observed similar changes in ZO-1 localization and expression in both experiments (**Figure 4G,**
299 **Figure S16B**), suggesting that AAA-10-mediated protection against intestinal permeability
300 occurred independent of weight loss. We also assessed the effect of AAA-10 or vehicle treatment
301 on normal chow-fed rats and found no significant differences between the groups in liver or kidney
302 function, body weight, or caloric intake (**Figure S17**), suggesting that AAA-10 itself was non-toxic
303 and that differences between groups are directly related to changes in intestinal BA composition.
304
305 We observed substantial histologic improvements in AAA-10-treated rats compared to vehicle-
306 treated controls. Specifically, AAA-10-treated rats exhibited significantly less hepatic steatosis,
307 lobular inflammation, and hepatocyte ballooning (**Figure 5B,C**). Consistent with these results, we
308 observed significant decreases in serum measures of hepatic inflammation including alanine
309 transaminase (ALT), aspartate transaminase (AST), and alkaline phosphatase (ALP) in AAA-10-
310 treated animals (**Figure 5D**). Finally, we observed decreased expression of pro-inflammatory and
311 pro-fibrotic genes in treated rats (**Figure 5E-F**). Together, our findings demonstrate that treatment
312 with the gut-restricted BSH inhibitor AAA-10 prevents the development of intestinal barrier
313 dysfunction and liver damage in CDAHFD-fed rats.
314
315 **Glyco-conjugated BAs form protective micelles in vitro**
316 While rodents possess only tauro-conjugated BAs, humans have a mixture of glyco- and tauro-
317 conjugated BAs, with the former dominating the pool in a ratio of 3:1 (**Figure 6A**) (Ridlon et al.,
318 2016). Further, microbial BSHs in the human intestine hydrolyze glyco-conjugated BAs to their
319 unconjugated forms (Ridlon et al., 2016). In order to test whether glyco-conjugated BAs are also
320 capable of forming micelles to sequester damaging unconjugated BAs, we determined critical
321 micelle concentrations (CMCs) of these BA populations as described above (**Figure 3A, B**). We
322 used an equimolar mix of (1) glyco-conjugated BAs (GCA, GDCA, GUDCA, GCDCA) or (2)

323 combined BA pools containing the same unconjugated BAs as used above (βMCA, CA, DCA,
324 UDCA, CDCA). Glyco-conjugated BAs exhibited a lower CMC than tauro-conjugated BAs (3.64
325 mM compared to 4.0 mM, respectively) (**Figure 6B, 3A**). Consistent with this observation and our
326 previous results, the CMC of the combined pool with glyco-conjugated BAs was 3.60 mM, while
327 the CMC of the combined pool of tauro-conjugated BAs was 4.2 mM (**Figure 6B, 3A**). We also
328 performed direct visualization of micelles using negative staining-electron microscopy. At 5 mM
329 concentration, no micelles were visible in the unconjugated BA pool while micelles were observed
330 in the glyco-conjugated and combined BA pools, consistent with our CMC determinations (**Figure**
331 **6C**). Together, these results suggest that glyco-conjugated BAs form micelles with unconjugated
332 BA species with equal to or greater efficiency than tauro-conjugated BAs.
333
334 Next, we sought to determine whether glyco-conjugated BAs would also protect the intestinal
335 epithelium from the damaging effects of unconjugated BAs. Using transwell-differentiated Caco-
336 2 cells, we found that unconjugated BA-induced epithelial monolayer damage was prevented by
337 addition of an equimolar concentration of glyco-conjugated BAs (**Figure 6D, E**). Glyco-conjugated
338 BAs alone did not disrupt monolayer integrity at any concentrations tested (**Figure 6E**). Similarly,
339 while unconjugated BAs were toxic to cells, equimolar addition of glyco-conjugated BAs
340 abrogated this effect (**Figure 6F**). Visualization of Caco-2 monolayers by hematoxylin and eosin
341 staining further confirmed that the toxic effects of unconjugated BAs were largely prevented by
342 addition of glyco-conjugated BAs (**Figure 6G**). Together, these results suggest that the
343 predominant human-derived glyco-conjugated BAs are also capable of sequestering
344 unconjugated BAs in micelles and protecting against epithelial permeability.

345 **Discussion**

346 In this study, we show that the onset of intestinal permeability is associated with decreased
347 intestinal and portal conjugated BA abundance in a diet-induced animal model of intestinal barrier
348 and liver damage. Furthermore, we demonstrate a mechanism by which conjugated BAs protect
349 intestinal epithelial barriers from damage in vitro via the sequestration of unconjugated BAs in
350 micelles. We then demonstrate that inhibition of gut bacterial BSH activity increases the
351 abundance of cecal conjugated BAs and prevents the development of intestinal barrier
352 dysfunction and liver inflammation in vivo. Finally, we show that both tauro-conjugated BAs and
353 glyco-conjugated BAs protect against unconjugated BA-induced epithelial barrier damage in vitro.
354 Because glyco- and tauro-conjugated BAs are the two conjugated BA forms found in humans, our
355 data suggest that conjugated BAs could be protective in the human gut.

356

357 The CDAHFD-fed rat model we utilized here to investigate pathogenic intestinal permeability is a
358 rodent model of chronic liver disease, including NAFLD and NASH progression (Longo et al.,
359 2020). Gut dysbiosis is a central feature of human NAFLD/NASH and has been proposed to
360 contribute to the development of these conditions (Boursier et al., 2016; Loomba et al., 2019).
361 Likewise, changes in serum BA profiles have been reported to correlate with disease severity in
362 NAFLD/NASH patients (Mouzaki et al., 2016; Nimer et al., 2020; Puri et al., 2018). However,
363 causal connections between gut bacteria, bile acid metabolism, and NAFLD/NASH in humans
364 have not yet been established. Our data raise the possibility that bacterial BA deconjugation may
365 be causally contributing to NAFLD/NASH progression. Indeed, we observed higher levels of BSH
366 activity in CDAHFD-treated rats compared to control animals. Future analyses will help uncover
367 whether correlations exist between fecal BA levels, BSH activity, and disease severity in human
368 patients. In addition, further studies in different animal models of pathogenic intestinal
369 permeability will reveal whether pharmacological BSH inhibition can prevent or ameliorate gut
370 barrier damage. Nonetheless, the finding that BSH inhibition prevents intestinal permeability and

371 liver damage in choline-deficient high fat diet-fed rodents, ~~a clinically relevant~~ a diet-induced
372 model of liver cirrhosis, suggests that modulating the in vivo BA pool by selective targeting of the
373 gut microbiota could be investigated as a treatment path for diseases characterized by intestinal
374 barrier disruption.
375
376 While intestinal barrier function has been implicated in the pathogenesis of a variety of diseases,
377 the molecular mechanisms that trigger or protect against intestinal permeability defects are
378 incompletely defined (Gupta et al., 2020). It has been shown that hydrophilic BAs, including
379 conjugated BAs, protect against cytotoxicity induced by hydrophobic bile acids, including many
380 unconjugated species (Araki et al., 2003; Di Ciaula et al., 2017; Hegyi, Maleth, Walters, Hofmann,
381 & Keely, 2018). However, prior to our work, the connection between the efficiency of micelle
382 formation by conjugated BAs and their ability to protect against unconjugated BA-induced
383 epithelial barrier damage had not been established. Here, we demonstrate that conjugated BAs
384 form micelles with unconjugated BAs at physiologically relevant concentrations in vitro. Micelle
385 formation leads to sequestration of hydrophobic, unconjugated BAs away from epithelial cells and
386 prevents cell death and tight junction dysfunction. These data demonstrate that distinct classes
387 of BAs elicit differential effects on intestinal epithelial permeability and delineate a mechanism by
388 which one of these classes, conjugated BAs, protects against epithelial damage in vitro. Whether
389 this mechanism is operable in vivo, however, is not yet known. Cholesterol, phospholipids, and
390 lipid hydrolysis products form mixed micelles with bile acids in bile and in the intestine (Hofmann,
391 1999). The presence of these other compounds could alter micelle formation and thus the
392 protective effects of conjugated BAs observed in vitro. To our knowledge, there are currently no
393 selective means of preventing micelle formation in vivo. Nonetheless, by inhibiting BSH activity
394 using AAA-10, we selectively shifted the in vivo BA pool toward conjugated BAs. These
395 experiments showed that, consistent with our in vitro data, conjugated BAs protect against
396 epithelial barrier damage in vivo.

397

398 The treatment of CDAHFD-fed rats with a gut-restricted BSH inhibitor not only prevented intestinal
399 permeability but also protected against the development of hepatic inflammation and steatosis.
400 The prevention of liver damage is likely multifactorial, and effects including decreased
401 translocation of bacterial products such as LPS as well as mild weight loss may contribute. The
402 weight loss observed is likely a result of BSH-dependent metabolic changes as we have
403 previously reported (Yao et al., 2018). Interestingly, germ-free mice, which lack BSH and therefore
404 have high levels of intestinal conjugated BAs, are resistant to development of hepatic steatosis
405 (Brandl & Schnabl, 2017). Moreover, colonization of germ-free mice on a HFD with BSH-
406 containing bacteria induces hepatic steatosis, a phenotype that can be prevented by deletion of
407 BSH (Yao et al., 2018). Our results here demonstrate that shifting the in vivo BA pool to enrich
408 for conjugated BAs can not only prevent pathogenic intestinal permeability but can also prevent
409 fat deposition and inflammation in the host liver.

410

411 Overall, our findings link changes in the intestinal BA pool, specifically conjugated BAs, with
412 maintenance of the intestinal epithelial barrier in vivo. Our study also reveals that bacterial BSH
413 enzymes regulate metabolites that control gut barrier integrity, thereby providing a potential
414 mechanistic link between the microbiome and the development of pathogenic intestinal
415 permeability. Our findings suggest that strategies that reduce BSH activity in the human gut
416 microbiome could be developed as novel paradigms to treat intestinal barrier dysfunction.

417

418 **Materials and Methods**

419

420 **Animals**. 8-week old Wistar rats were purchased from Charles River Laboratories (Wilmington,
421 MA) and housed in a specific pathogen-free environment (maximum four per cage). After 10 days
422 of acclimation, rats were initiated on either a control high-fat diet (60 kcal% fat; Research Diets

423 D12492) or CDAHFD (L-amino acid diet with 60 kcal% fat with 0.1% methionine without added
424 choline; Research Diets A06071302) *ad libitum* for either 48 hours or 1 week (either 7 or 8 days).
425 At time of sacrifice, rats were anesthetized using 100 mg/kg of ketamine and 10 mg/kg of xylazine
426 intraperitoneally followed by portal vein blood draw and terminal cardiac puncture.
427
428 **In vivo intestinal permeability assay**. Intestinal permeability was assessed by in vivo
429 fluorescein isothiocyanate (FITC)-dextran (FD4; Sigma-Aldrich, St. Louis, MO) permeability assay
430 as described previously (Woting & Blaut, 2018). Rats were fasted for 4 hours and then had blood
431 collected via tail vein puncture to assess background fluorescence. Rats were then gavaged with
432 0.4 mg/g body weight FITC-dextran 4kDa solution and blood was collected by terminal cardiac
433 puncture. Fluorescence intensity in the serum determined at 530 nm with excitation at 485 nm.
434 Relative fluorescence units determined by subtracting the PBS blank fluorescence from all
435 samples and then subtracting the pre-gavage fluorescence from the post-gavage fluorescence.
436 FITC-dextran concentrations were determined from a standard curve generated by serial dilutions
437 of FITC-dextran.
438
439 **AAA-10 treatment.** After initiation of CDAHFD diet, rats were split into two groups and were
440 gavaged twice a day with either 10 mg/kg of AAA-10 dissolved in 5% Captisol (Ligand, San Diego,
441 CA) and 5% DMSO in PBS or an equal volume of 5% Captisol and 5% DMSO in PBS.
442
443 **RNA extraction and RT-qPCR**. Total RNA was extracted from liver tissue using TRizol
444 (Invitrogen) according to the manufacturer's instructions and subsequently treated with DNase I
445 (Promega). cDNA was generated using the RevertAid First Strand cDNA Synthesis kit according
446 to manufacturer’s instructions (Thermo Fisher) and RT-qPCR was performed using the Power
447 SYBR Green Master Mix Kit (Thermo Fisher). Expression of *GAPDH* was used to standardize the
448 samples, and the results are expressed as a ratio relative to control. The genes for which

449 expression was determined and the primer sequences used in this study can be found in
450 **Supplementary Table 1**.
451
452 **Serum processing**. A cardiac terminal blood withdrawal was performed at the time of sacrifice.
453 Blood was allowed to clot for 2 hours at room temperature before centrifugation at 2,000 rpm for
454 10 minutes at 4°C. Serum was isolated and stored at -80°C. Biochemical markers of liver injury
455 were measured, including alkaline phosphatase, alanine aminotransferase, and aspartate
456 aminotransferase (DRI-CHEM 4000 Analyzer, Heska).
457
458 **Histology and immunofluorescence**. Formalin fixed paraffin embedded (FFPE) liver and ileum
459 tissue were sectioned at a thickness of 4 um. Slides were stained with hematoxylin and eosin at
460 the MGH Cytopathology Core. Slides were stained with Sirius Red for fibrosis staging. Slides
461 were blindly reviewed by a blinded pathologist to grade steatosis, inflammation, and fibrosis, using
462 criteria modeled on human NASH histologic scoring systems. Similarly, for intestine, slides were
463 evaluated for intestinal inflammation, epithelial hyperplasia, and goblet cell loss. For
464 immunofluorescence, sections were stained for ZO-1 (Abcam, Cambridge, MA) with detection by
465 appropriate secondary antibodies labeled with Alexa488 according to the manufacturer's
466 instructions. Hydroxyproline was quantified by high-performance liquid chromatography analysis
467 as previously described (Hutson, Crawford, & Sorkness, 2003).
468
469 For ZO-1 staining intensity measurement, images were processed and analyzed with Adobe
470 Photoshop CC software. Matched images were taken with the same exposure and were
471 processed and analyzed identically. The two-dimensional image intensities of ZO-1 fluorescence
472 (in pixels) of n=10 randomly selected cells in micrographs from each condition were measured
473 using Photoshop software (Adobe) and plotted in GraphPad Prism.
474

475 **LPS measurement assay**. Bacterial endotoxin measurement in portal serum samples was
476 performed using the Lonza Pyrogent turbidometric LAL assay kit (Lonza, N383) according to
477 manufacturer's instructions. Briefly, serial dilutions of prepared serum samples were made using
478 the LAL reagent water provided in the kit, followed by incubation for 15 min at 37ºC. Samples
479 were then mixed with the reconstituted Pyrogent reagent from the kit, followed by kinetic
480 turbidometric analyses in a SpectraMax M5 plate reader (Molecular Devices, San Jose, CA) at
481 the ICCB-Longwood Screening Facility at HMS. LPS amount in samples was deduced using
482 standard curves generated from stock provided in the kit and linear regression calculation.
483
484 **Bile acid analysis**. BA analyses were performed using a previously reported method (Yao et al.,
485 2018).
486 Reagents. Stock solutions of all bile acids were prepared by dissolving compounds in molecular
487 biology grade DMSO (VWR International). These solutions were used to establish standard
488 curves. HPLC grade solvents were used for preparing and running UPLC-MS samples.
489 Extraction. Rat cecal and human fecal samples (approximately 50 mg each) were collected in
490 pre-weighed lysis tubes. The lysis tubes contained ceramic beads to allow for homogenization
491 (Precellys lysing kit tough micro-organism lysing VK05 tubes for rat cecal and human fecal
492 samples; Bertin technologies, Montigny-le-Bretonneux, France). 400 µL of methanol (MeOH) was
493 added to rat cecal and human feces and the tubes were homogenized in a MagNA Lyser (6000
494 speed for 90 s*2, 7000 speed for 60 s). For portal venous samples, serum (100 µL) was collected
495 in eppendorf tubes, followed by addition of 100 µL MeOH. Samples were vortexed and frozen at
496 -20°C until further analysis. Cell culture media was diluted 1:1 in MeOH. All MeOH-extracted
497 samples were centrifuged at 4°C for 30 min at 15,000 rpm. The supernatant was diluted 1:1 in
498 50% MeOH/water and centrifuged again at 4°C for 30 min at 15000 rpm. The supernatant was
499 transferred into mass spec vials and injected into the UPLC-MS. Total bile acids were then
500 calculated by adding all detected and measured bile acids. The limits of detection for individual

501 bile acids have been described previously (Chaudhari, Harris, et al., 2021; Chaudhari, Luo, et al.,
502 2021; Yao et al., 2018).
503
504 **Caco-2 cell culture and differentiation**. Caco-2 cells were obtained from American Type Culture
505 Collection (Manassas, VA). Cells were maintained in Minimum Essential Medium (MEM) with
506 GlutaMAX and Earle's Salts (Gibco, Life Technologies, UK). Cell culture media were
507 supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL
508 streptomycin (GenClone). Cells were grown in FBS- and antibiotic-supplemented 'complete'
509 media at 37°C in an atmosphere of 5% CO_2. Undifferentiated Caco-2 cells were seeded in 24-
510 well plate transwells (0.4 µM pore size, Costar) at 200,000 cells per transwell. Media was changed
511 on days 4, 8, 12, 16, and 18 to differentiate Caco-2 cells in vitro (Lea, 2015). On day 21, fully
512 differentiated and polarized cells were used for permeability and toxicity assays.
513
514 **H&E staining and light microscopy of transwells**. Differentiated Caco-2 cells in transwells
515 were stained with H&E stain (Abcam, ab245880) according to manufacturer's instructions. Briefly,
516 transwells were washed with PBS, followed by staining with hematoxylin for 5 min. Transwells ere
517 rinsed in twice in dH_2O to remove excess stain, followed by addition of bluing reagent and
518 incubation for 10-15 sec. Two rinses in dH_2O was performed prior to addition of ethanol (Sigma)
519 to remove excess reagent. Ethanol was removed, and adequate Eosin was added for 2-3 min for
520 the counter stain. Transwells were dehydrated in ethanol 2-3 times, dried, and imaged using an
521 Evos XL Core microscope (Invitrogen) attached to camera at the ICCB-Longwood Screening
522 Facility at HMS. Cells were imaged from the top, and all images were taken at the same
523 magnification and light intensity. For each treatment, n=2 transwells were imaged, providing 4
524 images per transwell. Representative images are shown in **Figures 2, 3,** and **6**.
525

526 **In vitro bile acid treatments**. Caco-2 cells (undifferentiated in 96-well plates or day 21 to 25 of
527 differentiation in transwells) were treated with bile acid mixtures for 12-16 hours prior to assays.
528 Diluted stocks of bile acid standards or undiluted methanol-extracted cecal contents were added
529 in complete media. Transcytosis of bile acids was measured by drying basolateral media in a
530 speed vac followed by resuspending media in 1:1 methanol/water, transferred into mass
531 spectrometry vials and injected onto the ultra-high performance liquid chromatography-mass
532 spectrometer (UPLC-MS).
533
534 **Caco-2 permeability assay**. Epithelial integrity by FITC-dextran permeability assay was
535 performed as described previously (Chaudhari, Harris, et al., 2021). Briefly, differentiated Caco-
536 2 epithelial integrity was assayed by measuring passive diffusion of 4 kDa FITC-Dextran (Sigma
537 Aldrich) added at a concentration of 5 μM to the apical chamber in 100 μL PBS, while the
538 basolateral chamber contained 500 μL PBS. Diffusion from the apical to basolateral side was
539 measured by fluorescence reading in PBS on the basolateral side of the transwell system using
540 a SpectraMax M5 plate reader (Molecular Devices, San Jose, CA) at the ICCB-Longwood
541 Screening Facility. Fluorescence reading was normalized to the control.
542
543 **Caco-2 cell viability assay**. Caco-2 (undifferentiated in 96-well plates or day 21 to 25 of
544 differentiation in transwells) cell viability was measured using the MTT assay (Abcam, ab211091)
545 according to manufacturer's instructions. Cells were treated with compounds for 12-16 hours prior
546 to viability determination. Briefly, cell culture media was replaced with the MTT reagent and
547 incubated for 3 hours at 37ºC. Following incubation, the MTT reagent was replaced with the MTT
548 solvent and incubated for 15 min, followed by analysis in a SpectraMax M5 plate reader
549 (Molecular Devices, San Jose, CA) at the ICCB-Longwood Screening Facility at HMS.
550 Absorbance reading at 690 nm, as a measure of viability, was normalized to the control.
551

552 **Critical micelle concentration (CMC) assay**. CMC determination of groups of bile acids was
553 performed using a previously described assay using coumarin 6 as a fluorescent probe with minor
554 adaptations (Fluksman & Benny, 2019). Briefly, 6 mM coumarin 6 (Sigma) in dichloromethane
555 (Sigma) was added to Eppendorf tubes and allowed to evaporate for 30 min in a chemical hood.
556 400 µL of equimolar mixtures of bile acids at various concentrations to be tested were added to
557 tubes and rotated overnight at room temperature in the dark (unconjugated BAs: beta-muricholic
558 acid [βMCA], cholic acid [CA], deoxycholic acid [DCA], ursodeoxycholic acid [UDCA],
559 chenodeoxycholic acid [CDCA]); tauro-conjugated BAs: tauro-beta-muricholic acid [TβMCA],
560 tauro-cholic acid [TCA], tauro-deoxycholic acid [TDCA], tauro-ursodeoxycholic acid [TUDCA],
561 tauro-chenodeoxycholic acid [TCDCA]); glyco-conjugated BAs: glyco-cholic acid [GCA], glyco-
562 deoxycholic acid [GDCA], glyco-ursodeoxycholic acid [GUDCA], glyco-chenodeoxycholic acid
563 [GCDCA]). The next day, 200 µL of the solution was transferred to black 96 well plates (Costar),
564 and fluorescence intensity at 480/530 was measured using a SpectraMax M5 plate reader
565 (Molecular Devices, San Jose, CA) at the ICCB-Longwood Screening Facility. Fluorescence
566 intensity was plotted against the logarithm of the corresponding concentration and the CMC was
567 determined by the intersection of the two tangents created in the graph.
568
569 **Electron microscopy**. Caco-2 cells seeded on transwells were washed in PBS prior to fixation
570 in FGOP (Formaldehyde-Glutaraldehyde-Picric acid) fixative. Samples were fixed overnight in a
571 mixture of 1.25% formaldehyde, 2.5 % glutaraldehyde, and 0.03% picric acid in 0.1 M sodium
572 cacodylate buffer, pH 7.4. FGP fixative was diluted in PBS 1:1 before applying to the apical and
573 basolateral chamber of transwells. Fixed samples were stored at 4°C until further processing and
574 imaging by the Electron Microscopy Core at Harvard Medical School. All electron microscopy
575 imaging was performed by a blinded investigator.
576

577 For transmission electron microscopy, fixed tissues were washed with 0.1M sodium cacodylate
578 buffer and post fixed with 1% osmium tetroxide/1.5% potassium ferrocyanide (in H_2O) for 2 hours.
579 Samples were then washed in a maleate buffer and post fixed in 1% uranyl acetate in maleate
580 buffer for 1 hour. Tissues were then rinsed in ddH_2O and dehydrated through a series of ethanol
581 (50%, 70%, 95%, (2x)100%) for 15 minutes per solution. Dehydrated tissues were put in
582 propylene oxide for 5 minutes before they were infiltrated in epon mixed 1:1 with propylene oxide
583 overnight at 4C. Samples were polymerized in a 60ºC oven in epon resin for 48 hours. They were
584 then sectioned into 80nm thin sections and imaged on a JEOL 1200EX Transmission Electron
585 Microscope.
586
587 For negative staining for visualization of micelles, the sample was diluted in water and adsorbed
588 onto a glow-discharged carbon grid. Once the specimen was adsorbed on to the film surface, the
589 excess liquid was blotted off using a filter paper (Whatman #1) and the grid was floated on a small
590 drop (~5 µl) of staining solution (0.75% uranyl acetate). After 20 seconds the excess stain was
591 blotted off and the sample was air dried briefly before it was examined in the TEM.
592
593 **BSH activity assay.** BSH activity was quantified using a modified version of a previously
594 described method (Adhikari et al., 2020). Briefly, fresh cecal contents (approximately 20 mg) were
595 diluted in PBS to obtain a concentration of 1 mg/mL 100 µM glycochenodeoxycholic acid-d4
596 (GCDCA-d4) was added to the mixture and incubated at 37ºC for 30 minutes, then frozen in dry
597 ice for 5 minutes and stored at -80ºC until further analysis. On thawing, the mixture was diluted
598 with an equal volume of methanol and the slurry was centrifuged at 12,500 x g for 10 minutes.
599 The supernatant was removed into a clean Eppendorf tube and centrifuged again. The
600 supernatant was transferred to mass spectrometry vials and samples were analyzed as described
601 in Bile Acid Analysis in Supplementary Information.
602

603 **Statistical Analyses**. Data was quantified using software linked to indicated instruments and
604 plotted in GraphPad Prism 7. Statistical analyses were performed using GraphPad Prism and
605 Microsoft Excel software. Statistical significance was assessed using Student's or Welch's t tests,
606 one-way or two-way ANOVAs followed by multiple comparisons tests, and Mann-Whitney tests
607 wherever appropriate. **Figures 1E, F, 4G,** and **S16**: Intestines from all animals were stained, n=10
608 intestinal cell images per group were analyzed. For ZO-1 intensity, one-way ANOVA followed by
609 Tukey's multiple comparison test, for %ZO-1 membrane intensity and DAPI intensity, two-tailed
610 Welch's t test. Figures 2A, B: one-way ANOVA followed by Dunnett's multiple comparison test for
611 treatment vs. DMSO, one-way ANOVA followed by Tukey's multiple comparison test for
612 comparing treatments. **Figures 2D, E, 3D, E, G, 6E, F**: 2-way ANOVA followed by Sidak's multiple
613 comparisons test.
614
615 **Ethics.** Animals received humane care per criteria outlined in the Guide for the Care and Use of
616 Laboratory Animals by the National Academy of Sciences (National Institutes of Health publication
617 86-23, revised 1985) and in accordance with the Massachusetts General Hospital Institutional
618 Animal Care and Use Committee guidelines (Protocol 2007N000113).
619

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644
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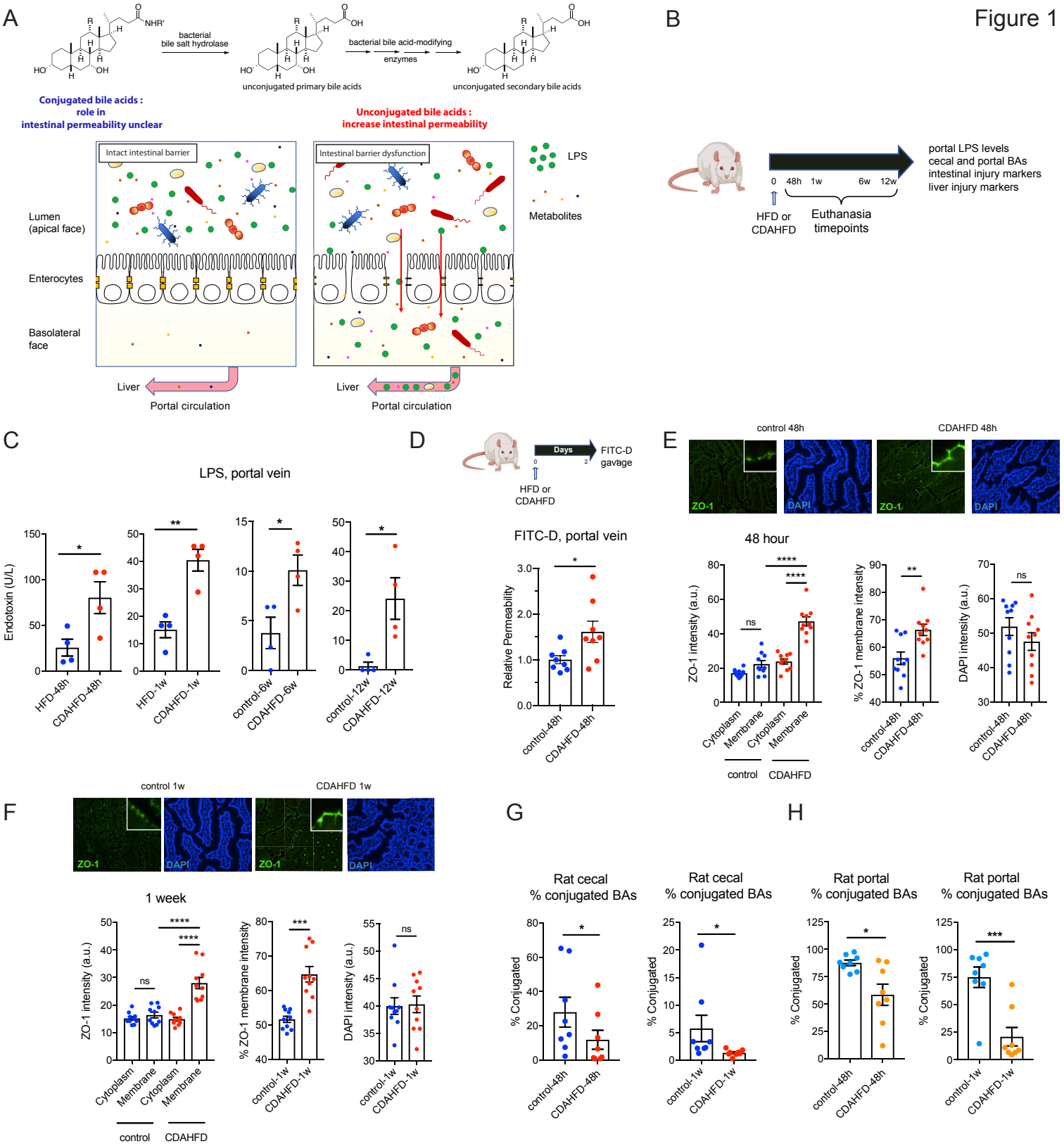
840

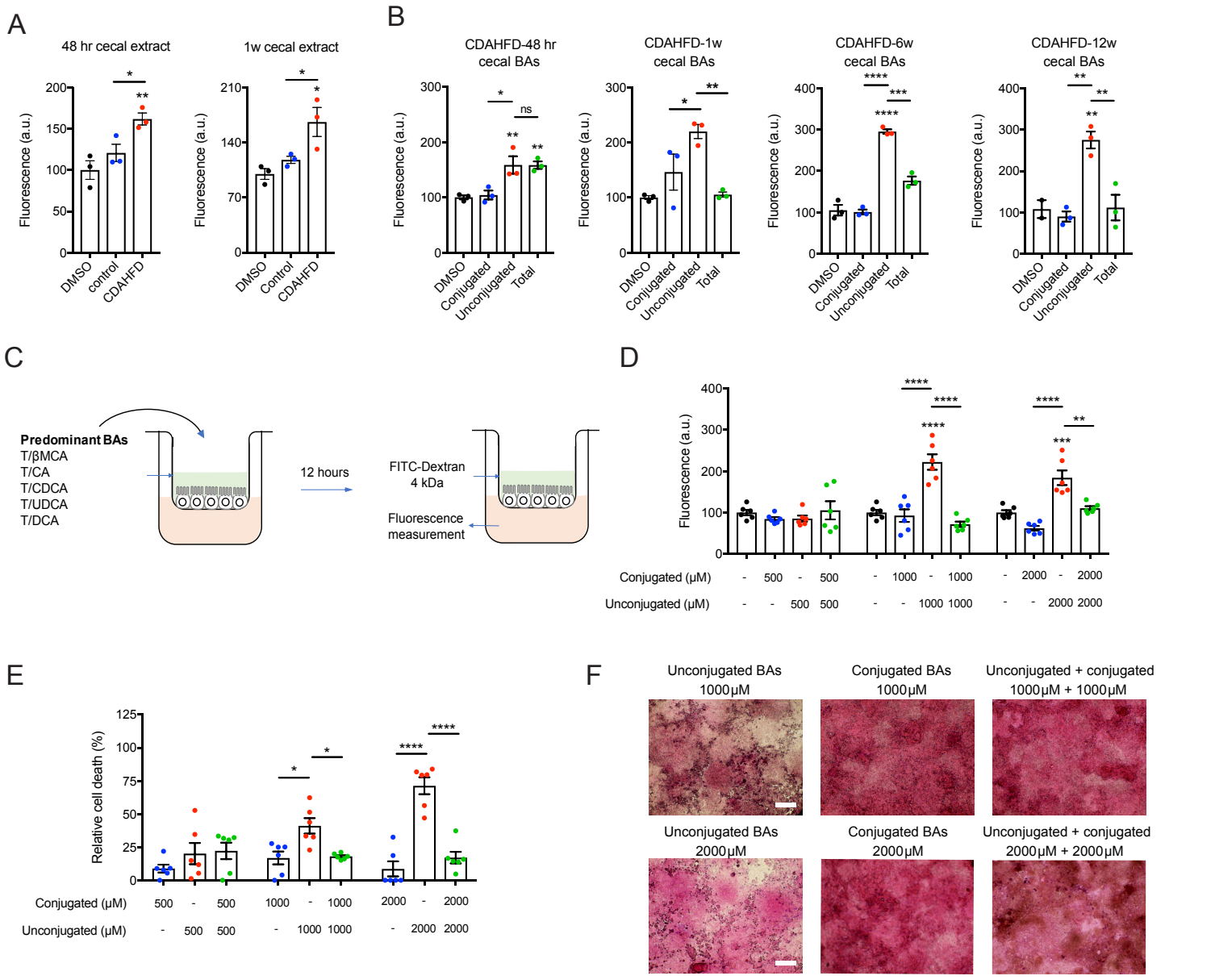
Figure 1


A
bacterial bile salt hydrolase
bacterial bile acid-modifying enzymes
unconjugated primary bile acids
unconjugated secondary bile acids
Conjugated bile acids : role in intestinal permeability unclear
Unconjugated bile acids : increase intestinal permeability
Intact intestinal barrier
Intestinal barrier dysfunction
LPS
Metabolites
Lumen (apical face)
Enterocytes
Basolateral face
Liver
Portal circulation
B
portal LPS levels
cecal and portal BAs
intestinal injury markers
liver injury markers
HFD or CDAHFD
Euthanasia timepoints
C
LPS, portal vein
Endotoxin (U/L)
HFD-48h
CDAHFD-48h
HFD-1w
CDAHFD-1w
control-6w
CDAHFD-6w
control-12w
CDAHFD-12w
D
Days
FITC-D gavage
HFD or CDAHFD
FITC-D, portal vein
Relative Permeability
control-48h
CDAHFD-48h
E
control 48h
CDAHFD 48h
ZO-1
DAPI
48 hour
ZO-1 intensity (a.u.)
% ZO-1 membrane intensity
DAPI intensity (a.u.)
Cytoplasm
Membrane
control
CDAHFD
F
control 1w
CDAHFD 1w
1 week
control-1w
CDAHFD-1w
G
Rat cecal % conjugated BAs
% Conjugated
H
Rat portal % conjugated BAs

841

842 **Figure 1. CDAHFD-fed rats develop increased intestinal permeability at early timepoints**
843 **coinciding with decreased conjugated BA abundance.** (A) Human gut bacterial enzymes
844 transform conjugated primary BAs into unconjugated secondary BAs. While hydrophilic
845 unconjugated BAs damage epithelial integrity, the roles of unconjugated BAs and bacterial BSHs
846 in intestinal membrane integrity are unclear. (B) Schematic of rat intestinal permeability
847 timecourse experiment. A choline-deficient, L-amino acid defined, high-fat diet was fed to rats to
848 induce intestinal permeability and liver damage. High fat diet-fed rats served as controls. Rats
849 were euthanized at 48 hours, 1 week, 6 week, or 12 week timepoints. Tissues and blood were
850 collected for metabolite quantification and evaluation of intestinal and liver injury markers. (C)
851 Portal venous levels of lipopolysaccharide (LPS) were significantly increased in CDAHFD-fed rats
852 compared to control rats after 48h, 1w, 6w, and 12w on diet (n=4 per group, two-tailed Welch's t
853 test). (D) CDAHFD-fed rats developed increased intestinal permeability after 48h of diet.
854 Measurement of FITC-Dextran levels in systemic circulation after gavage in control and CDAHFD-
855 fed rats (n=8 per group, two-tailed Welch's t test). (E-F) CDAHFD induced increased expression
856 and apical membrane localization of ZO-1 at early timepoints. ZO-1 immunofluorescence staining
857 of ileum from control and CDAHFD-fed rats at 48h (E) and 1w (F) timepoints (n=10 intestinal cells
858 quantified per group, see Methods for statistical analyses). (G, H) Conjugated BA abundance was
859 reduced at early timepoints (48h, 1w) in the cecum (G) and portal vein (H) of CDAHFD-fed rats
860 as determined by UPLC-MS BA analysis from control and CDAHFD-fed animals (n=8 per group,
861 two-tailed Welch's t test, see Supplementary Information for concentrations of total and individual
862 BAs in cecal contents and portal vein at 48h, 1w).
863 See Methods for statistical analyses. *p<0.05, **p<0.005, ***p<0.001, ****p<0.0001. Bars
864 represent mean ± SEM.
865

Figure 2


A
48 hr cecal extract
1w cecal extract
Fluorescence (a.u.)
DMSO
control
CDAHFD
Control
B
CDAHFD-48 hr cecal BAs
CDAHFD-1w cecal BAs
CDAHFD-6w cecal BAs
CDAHFD-12w cecal BAs
Conjugated
Unconjugated
Total
ns
C
Predominant BAs
T/βMCA
T/CA
T/CDCA
T/UDCA
T/DCA
12 hours
FITC-Dextran 4 kDa
Fluorescence measurement
D
Conjugated (μM)
Unconjugated (μM)
E
Relative cell death (%)
F
Unconjugated BAs 1000μM
Conjugated BAs 1000μM
Unconjugated + conjugated 1000μM + 1000μM
Unconjugated BAs 2000μM
Conjugated BAs 2000μM
Unconjugated + conjugated 2000μM + 2000μM
G
Unconjugated BAs - 2mM
Conjugated BAs - 2mM
Unconjugated (1 mM) + conjugated (1 mM)

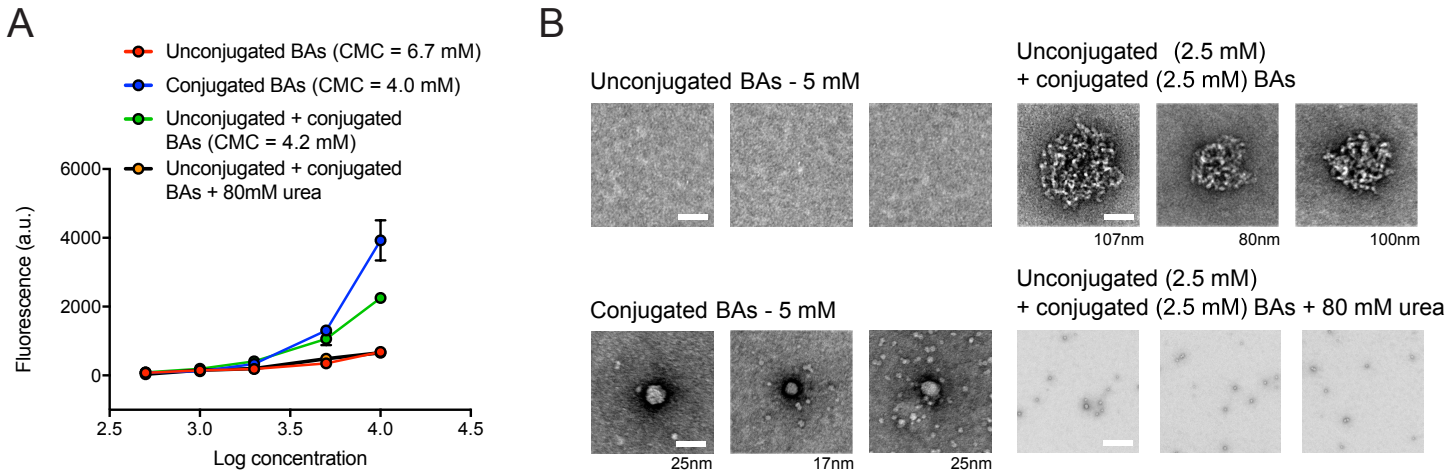
866

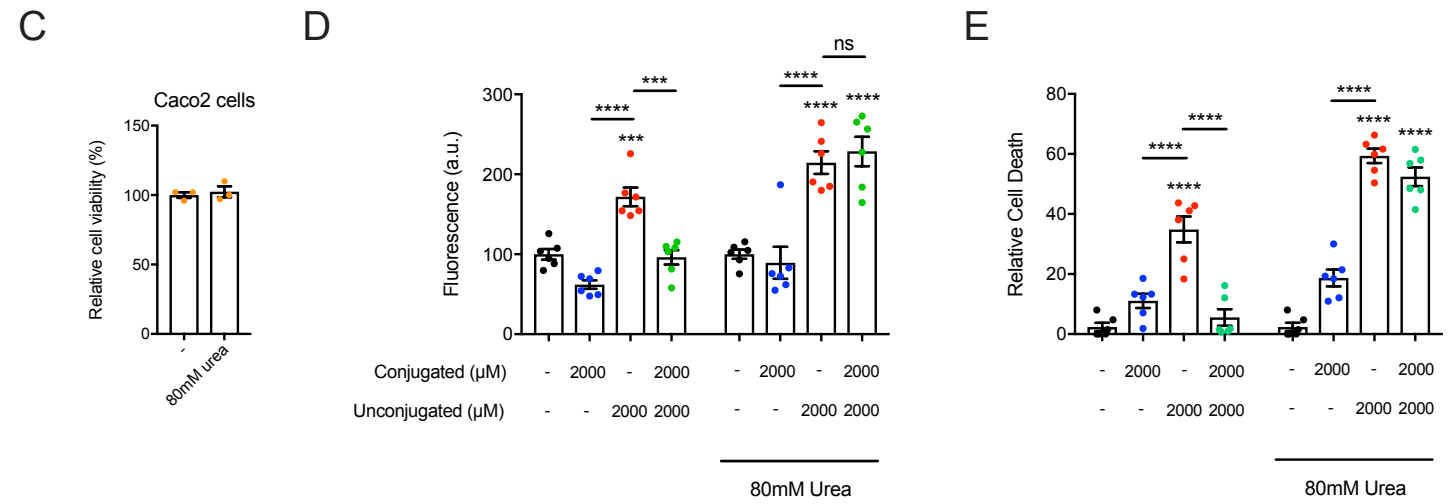
867 **Figure 2. Conjugated BAs protect epithelial monolayers from unconjugated BA-induced**
868 **permeability.**

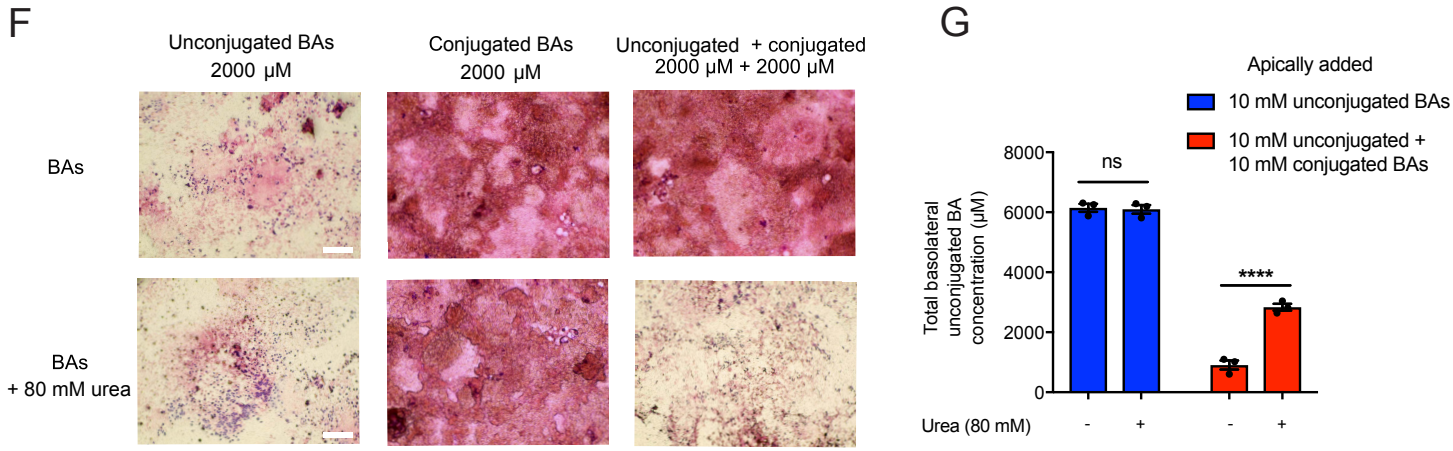
869 (A) Cecal extracts from CDAHFD-fed rats at early timepoints caused increased epithelial
870 permeability. Caco-2 monolayer permeability after exposure to purified cecal extracts from control
871 and CDAHFD-fed rats at indicated timepoints as measured by FITC-Dextran passage into the
872 basolateral chamber over 12 hours (n=3 per group, see Methods for statistical analyses). (B)
873 Conjugated BAs alone did not increase Caco-2 monolayer permeability at concentrations found
874 in the cecum of CDAHFD rats, and when combined with unconjugated BAs, conjugated BAs
875 protected against unconjugated BA-induced permeability (n=3 per group, see Methods for
876 statistical analyses). (C) Schematic of in vitro permeability experiment. Conjugated BAs
877 (equimolar concentrations of TβMCA, TCA, TCDCA, TUDCA, TDCA), unconjugated BAs
878 (equimolar concentrations of βMCA, CA, CDCA, UDCA, DCA), and combined BA pools
879 (equimolar concentrations of both pools) were added to the apical chamber of a transwell system
880 containing a Caco-2 monolayer and incubated for 12 hours before addition of FITC-Dextran 4kDa
881 and fluorescence measurement from basolateral chamber. (D) Conjugated BAs protected against
882 unconjugated BA-induced Caco-2 monolayer permeability at physiologic concentrations (n=6 per
883 group, see Methods for statistical analyses). (E) Conjugated BAs protected epithelial monolayers
884 from unconjugated BA-induced cell death. Cell viability of Caco-2 cells measured by MTT assay
885 (n=6 per group, see Methods for statistical analyses). (F) Conjugated BAs protected the physical
886 integrity of epithelial monolayers from unconjugated BA-induced damage. Representative light
887 microscopy images of H&E-stained Caco-2 monolayers after exposure to BA pools. Scale bar=20
888 μm. (G) Conjugated BAs prevented the development of unconjugated BA-induced tight junction
889 dilatation. Representative TEM images of Caco-2 cells from transwells after exposure to BA pools
890 at indicated concentrations. The white arrow points to tight junction dilatation. Scale bar=500 nm.

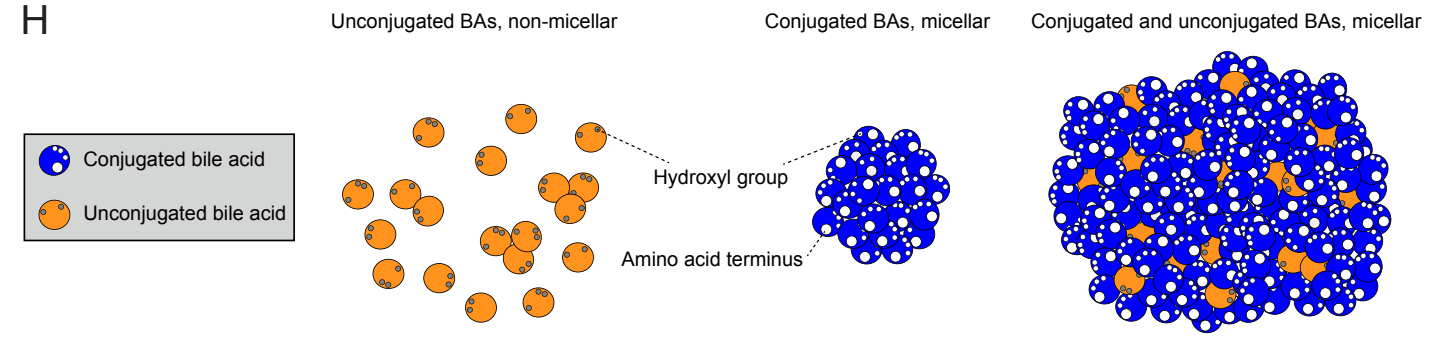
891 see Supplementary Information for additional representative TEM images. Unless otherwise
892 specified, all experiments were performed in triplicate. See Methods for statistical analyses. Data
893 not marked with asterisk(s) are not significant. *$p<0.05$, **$p<0.005$, ***$p<0.001$, ****$p<0.0001$.
894 Bars represent mean ± SEM.
895

Figure 3


A
Unconjugated BAs (CMC = 6.7 mM)
Conjugated BAs (CMC = 4.0 mM)
Unconjugated + conjugated BAs (CMC = 4.2 mM)
Unconjugated + conjugated BAs + 80mM urea
Fluorescence (a.u.)
Log concentration
B
Unconjugated BAs - 5 mM
Conjugated BAs - 5 mM
Unconjugated (2.5 mM) + conjugated (2.5 mM) BAs
Unconjugated (2.5 mM) + conjugated (2.5 mM) BAs + 80 mM urea
107nm
80nm
100nm
25nm
17nm
25nm


C
Caco2 cells
Relative cell viability (%)
80mM urea
D
Fluorescence (a.u.)
Conjugated (μM)
Unconjugated (μM)
80mM Urea
ns
E
Relative Cell Death
80mM Urea


F
Unconjugated BAs 2000 μM
Conjugated BAs 2000 μM
Unconjugated + conjugated 2000 μM + 2000 μM
BAs
BAs + 80 mM urea
G
Apically added
10 mM unconjugated BAs
10 mM unconjugated + 10 mM conjugated BAs
Total basolateral unconjugated BA concentration (μM)
ns
Urea (80 mM)


H
Unconjugated BAs, non-micellar
Conjugated BAs, micellar
Conjugated and unconjugated BAs, micellar
Conjugated bile acid
Unconjugated bile acid
Hydroxyl group
Amino acid terminus

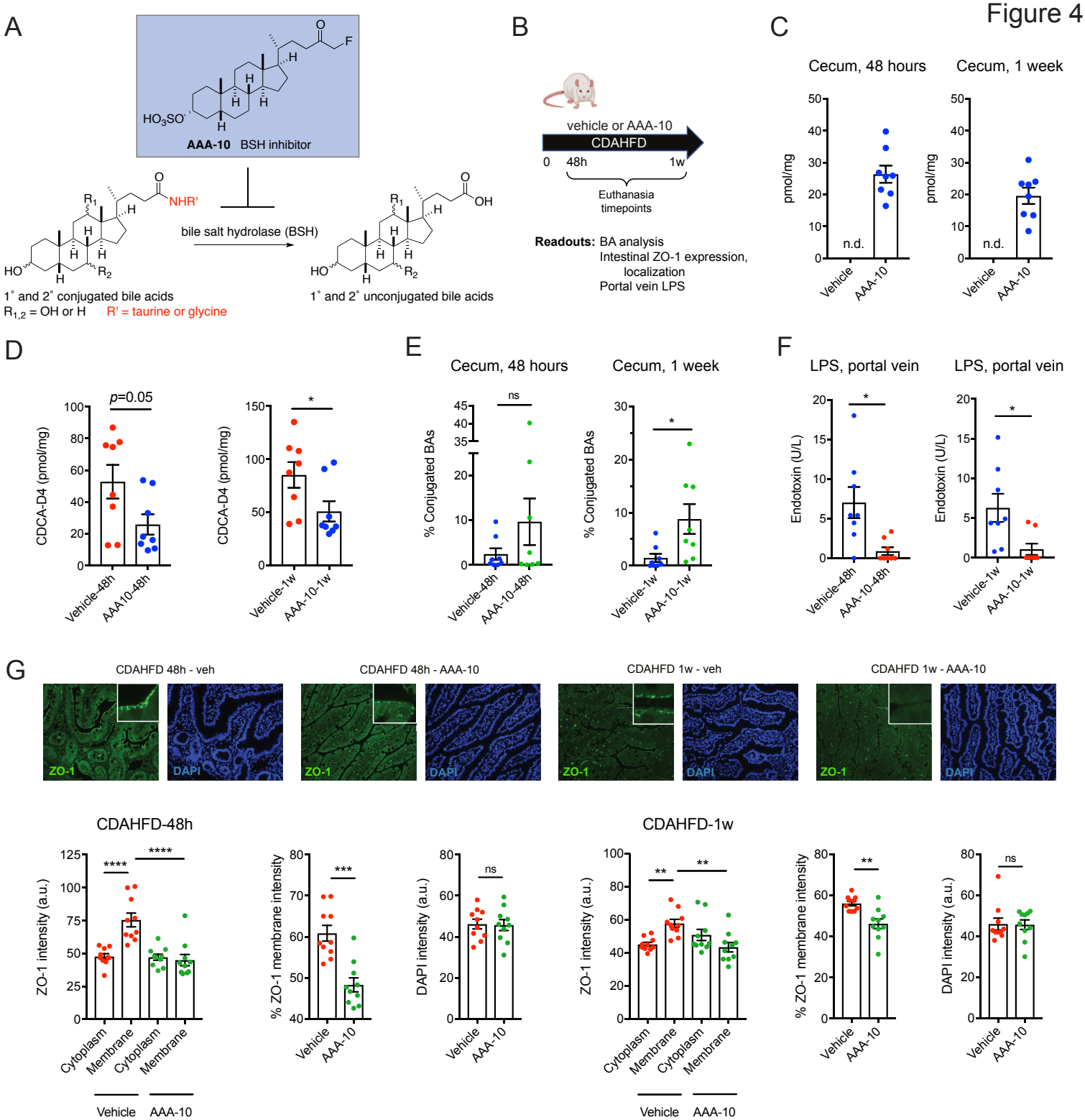
896

897 **Figure 3. Conjugated and unconjugated BAs form micelles that sequester unconjugated**
898 **BAs and prevent epithelial damage.**

899 (A) Conjugated BAs alone (equimolar concentrations of TβMCA, TCA, TCDCA, TUDCA, TDCA)
900 and conjugated BAs combined with unconjugated BAs (equimolar concentrations of conjugated
901 BAs with βMCA, CA, CDCA, UDCA, DCA) exhibited lower CMCs than unconjugated BAs alone.
902 Micelle formation was disrupted after addition of urea (80 mM) (n=3 per group). (B) EM images
903 of micelles formed from BA pools at indicated concentrations. While detectable micelles were
904 visible in the conjugated BAs alone or unconjugated plus conjugated BA pools, no detectable
905 micelles were visible in the unconjugated BAs or unconjugated plus conjugated BAs plus urea
906 (80 mM) pools. Scale bar=50 nm, diameter of micelle indicated beneath each image (n=3 images
907 per group). (C) MTT cell viability assay of Caco-2 cells in the presence or absence of 80 mM urea
908 indicated that urea did not impact Caco-2 cell viability (n=3 per group, not significant by two-tailed
909 Welch's t test). (D) Micelle formation was necessary for the protective effect of conjugated BAs
910 on epithelial permeability. Permeability measured by fluorescence (FITC-Dextran) in basolateral
911 chamber in the presence or absence of 80 mM urea (n=6 per group, see Methods for statistical
912 analyses). (E) Micelle formation was necessary for the protective effect of conjugated BAs on cell
913 viability. Caco-2 cell viability measured by MTT assay in the presence or absence of urea (n=6
914 per group, see Methods for statistical analyses). (F) Micelle formation was necessary for the
915 protective effect of conjugated BAs on epithelial layer integrity. Representative light microscopy
916 images of H&E-stained Caco-2 monolayers in transwells in the presence or absence of urea. (G)
917 Addition of urea to a mixed BA pool led to increased unconjugated BA passage across a Caco-2
918 monolayer. Quantification of basolateral concentrations of unconjugated BAs by UPLC-MS (n=3
919 per group, see Methods for statistical analyses). (H) Model for the sequestration of unconjugated
920 BAs in micelles by conjugated BAs. BAs shown in cross-section and micellar shape depictions

921 based on previous micelle models (Carey & Small, 1972; Faramarzi et al., 2017). While
922 unconjugated BAs are largely non-micellar in solution, both conjugated BAs and mixtures of
923 unconjugated and conjugated BAs form micelles. Unless otherwise specified, all experiments
924 were performed in triplicate. See Methods for statistical analyses. Data not marked with
925 asterisk(s) are not significant. *$p<0.05$, **$p<0.005$, ***$p<0.001$, ****$p<0.0001$. Bars represent
926 mean ± SEM.
927

Figure 4

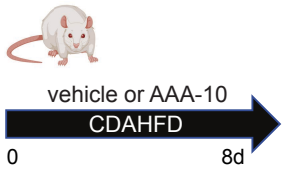

A
AAA-10 BSH inhibitor
bile salt hydrolase (BSH)
1˚ and 2˚ conjugated bile acids
R1,2 = OH or H
R' = taurine or glycine
1˚ and 2˚ unconjugated bile acids
B
vehicle or AAA-10
CDAHFD
0
48h
1w
Euthanasia timepoints
Readouts: BA analysis
Intestinal ZO-1 expression, localization
Portal vein LPS
C
Cecum, 48 hours
Cecum, 1 week
pmol/mg
n.d.
Vehicle
AAA-10
D
p=0.05
CDCA-D4 (pmol/mg)
Vehicle-48h
AAA10-48h
Vehicle-1w
AAA-10-1w
E
Cecum, 48 hours
Cecum, 1 week
% Conjugated BAs
ns
Vehicle-48h
AAA-10-48h
Vehicle-1w
AAA-10-1w
F
LPS, portal vein
Endotoxin (U/L)
Vehicle-48h
AAA-10-48h
Vehicle-1w
AAA-10-1w
G
CDAHFD 48h - veh
CDAHFD 48h - AAA-10
CDAHFD 1w - veh
CDAHFD 1w - AAA-10
ZO-1
DAPI
CDAHFD-48h
CDAHFD-1w
ZO-1 intensity (a.u.)
% ZO-1 membrane intensity
DAPI intensity (a.u.)
Cytoplasm
Membrane
Vehicle
AAA-10

928

929 **Figure 4. BSH inhibition increases intestinal conjugated BAs and prevents intestinal**
930 **barrier dysfunction in CDAHFD-fed rats.** (A) Schematic for the inhibition of bacterial BSH
931 activity by the covalent pan-BSH inhibitor AAA-10 (Adhikari et al., 2021; Adhikari et al., 2020). (B)
932 Schematic of rat intestinal permeability prevention experiment. Fats were fed CDAHFD and
933 administered either AAA-10 (10 mg/kg/dose) or vehicle twice daily by gavage. Rats were
934 euthanized at 48 hours or 1 week. Tissues and blood were collected for metabolite quantification
935 and evaluation of intestinal injury markers (n=8 per for all timepoints). (C) UPLC-MS analysis of
936 cecal contents of vehicle- and AAA-10-treated animals. Cecal AAA-10 levels were within the
937 concentration range previously shown to be effective for inhibiting BSH activity (Adhikari et al.,
938 2021; Adhikari et al., 2020). (D) Cecal BSH activity was reduced after 1 week of AAA-10
939 treatment. BSH activity was determined by quantifying conversion of GCDCA-d4 to CDCA-d4 in
940 feces from vehicle- or AAA-10-treated animals (two-tailed Welch's t-test). (E) Cecal conjugated
941 BA abundance was significantly increased in AAA-10-treated rats after 1 week (two-tailed Welch's
942 t test, see Supplementary Information for concentrations of total and individual BAs in cecal
943 contents at 1w). (F) Portal venous LPS levels were reduced in AAA-10 treated rats after 48h and
944 1w of AAA-10 treatment (two-tailed Welch's t test). (G) AAA-10 treatment prevented aberrant ZO-
945 1 subcellular localization. ZO-1 immunofluorescence and DAPI counterstaining of rat ileum from
946 vehicle and AAA-10 treated CDAHFD-fed rats at 48h and 1w (n=10 intestinal cells quantified per
947 group, see Methods for statistical analyses). Data not marked with asterisk(s) are not significant.
948 *p<0.05, **p<0.005, ***p<0.005, ****p<0.0001. Bars represent mean ± SEM.
949

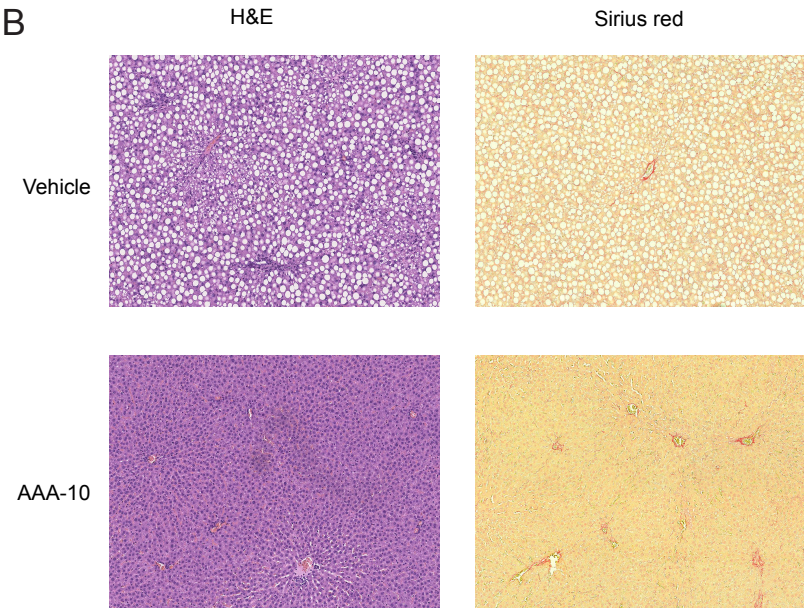
Figure 5

A

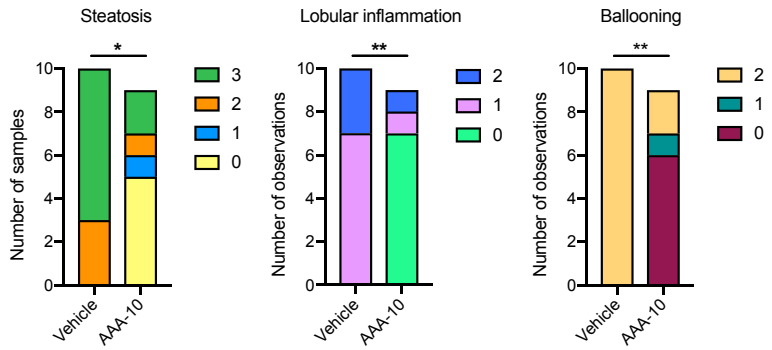


Readouts: Liver histology
Serum biomarkers
qPCR (liver)
BA analysis

B



C



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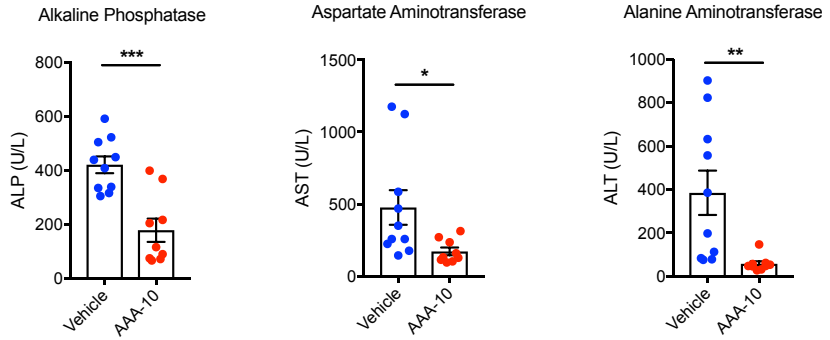

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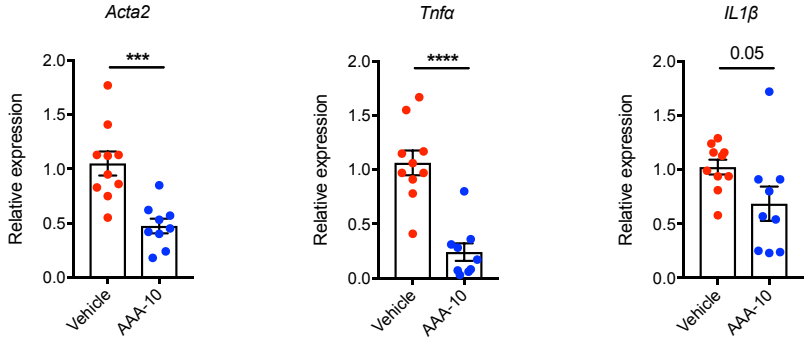

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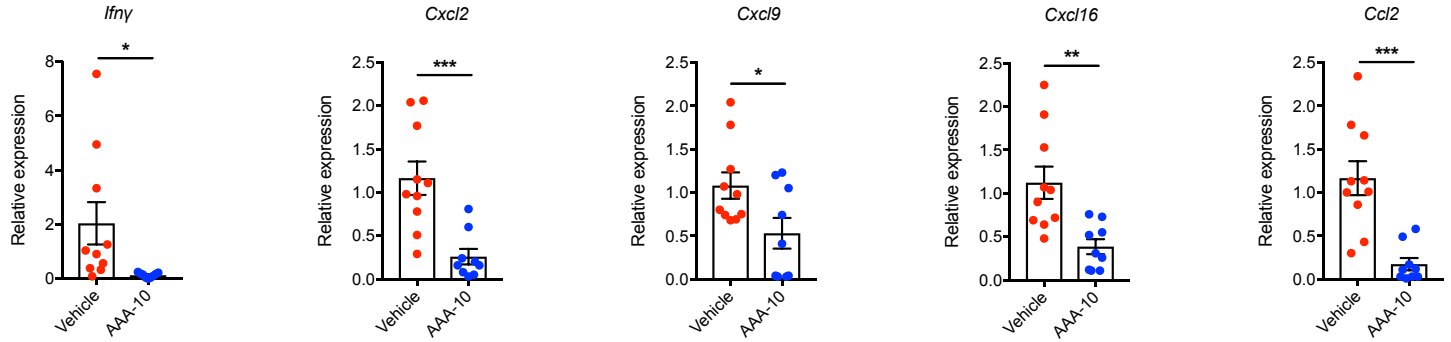


950

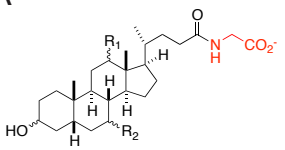
951 **Figure 5. BSH inhibition prevents hepatic inflammation in CDAHFD-fed rats.**

952 (A) Schematic of rat liver damage prevention experiment. Rats were fed CDAHFD and
953 administered either AAA-10 (10 mg/kg/dose) or vehicle twice daily by gavage. Rats were
954 euthanized after 8 days. Tissues and blood were collected for metabolite quantification and
955 evaluation of liver injury markers (n=10 in vehicle group, n=9 in AAA-10 group for all timepoints).
956 (B) AAA-10 treatment prevented development of hepatic steatosis and inflammation in CDAHFD-
957 fed rats. Representative H&E staining of liver tissue is shown. (C) AAA-10 treatment prevented
958 development of hepatic inflammation. Histologic scoring of steatosis, hepatocyte ballooning, and
959 lobular inflammation in vehicle- and AAA-10-treated animals. Mann-Whitney test. (D) Serum ALT,
960 AST, and ALP levels were decreased in AAA-10-treated compared to vehicle-treated rats, two-
961 tailed Welch's t test. (E, F) AAA-10 treatment attenuated hepatic gene expression of pro-
962 inflammatory and pro-fibrotic genes in CDAHFD-fed rats as determined by RT-qPCR analysis of
963 indicated genes. Two-tailed Welch's t test. *$p<0.05$, **$p<0.005$, ***$p<0.0005$, ****$p<0.0001$. Bars
964 represent mean ± SEM.

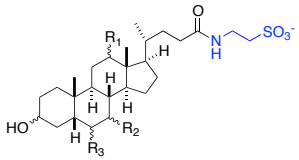
965

Figure 6

A

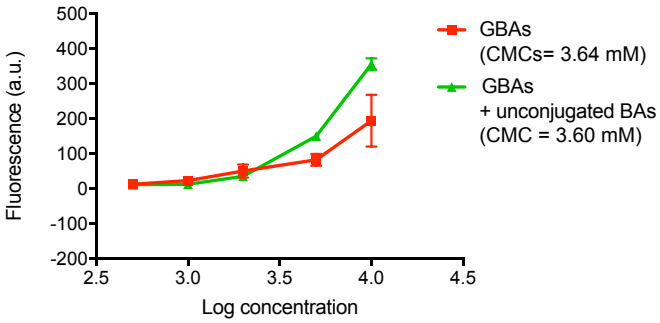


Glyco-conjugated bile acids (GBAs)
Predominant bile acids in humans
Not found in rodents



Tauro-conjugated bile acids (TBAs)
Predominant bile acids in rodents
Also found in humans

B



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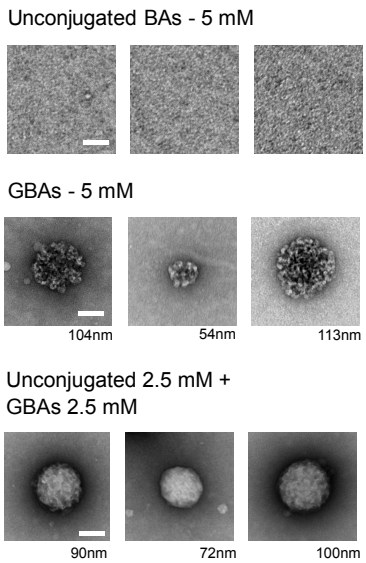

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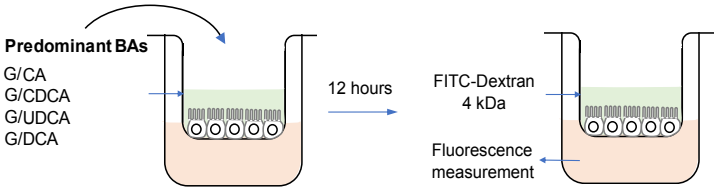

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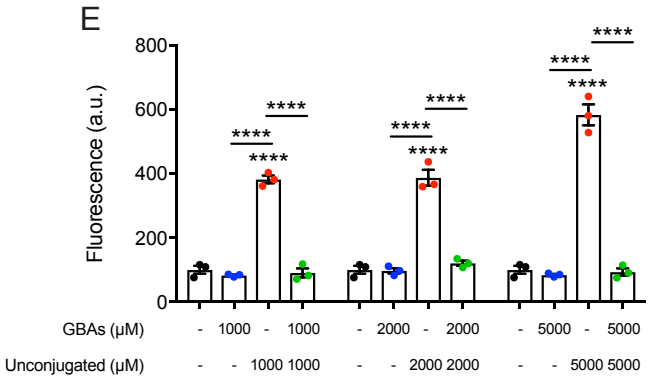

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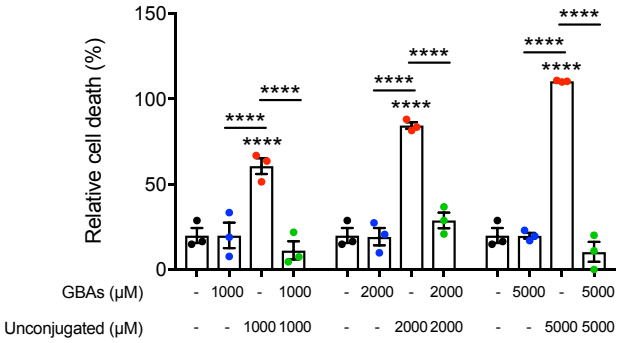

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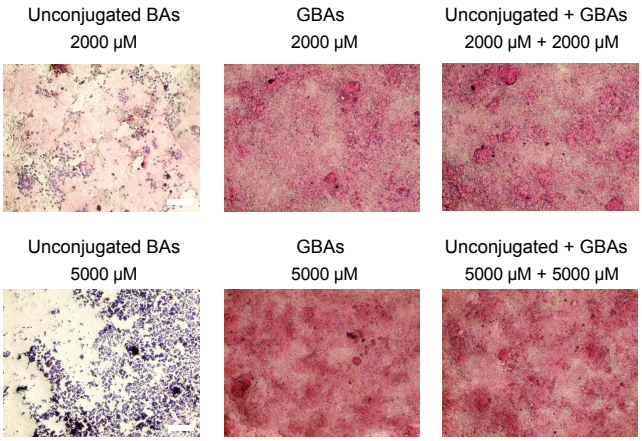

966

967 **Figure 6. Predominant human glyco-conjugated BAs sequester unconjugated BAs in**
968 **micelles and prevent epithelial damage in vitro.** (A) Structures of glyco- and tauro-conjugated
969 BAs (GBAs and TBAs, respectively). While TBAs are found in both rodents and humans, GBAs
970 are the predominant conjugated BAs in humans and are not present in rodents. (B) Pools of GBAs
971 alone (equimolar concentrations of GCA, GCDCA, GUDCA, GDCA) and GBAs plus unconjugated
972 BAs (equimolar concentrations of glyco-conjugated BAs with βMCA, CA, CDCA, UDCA, DCA)
973 exhibited CMCs of 3.64 mM and 3.60 mM, respectively, indicating efficient micelle formation (n=3
974 per group). (C) EM images of micelles formed from BA pools at indicated concentrations. Similar
975 to TBAs (see Figure 3), while detectable micelles were visible in the GBAs alone or unconjugated
976 BAs plus GBA pools, no detectable micelles were visible in the unconjugated BAs pool. Scale
977 bar=50 nm, diameter of micelle indicated beneath each image. (D) Schematic of in vitro
978 permeability experiment. Equimolar concentrations of GBAs, unconjugated BAs, and combined
979 BA pools were added to the apical chamber of transwell system containing Caco-2 monolayer
980 and incubated for 12 hours before addition of FITC-Dextran 4kDa and fluorescence measurement
981 from basolateral chamber. (E) GBAs protected against unconjugated BA-induced Caco-2
982 monolayer permeability at physiologic concentrations (n=3 per group, see Methods for statistical
983 analyses). (F) GBAs protected epithelial monolayers from unconjugated BA-induced cell death.
984 Cell viability of Caco-2 cells measured by MTT assay (n=3 per group, see Methods for statistical
985 analyses). (G) GBAs protected the physical integrity of epithelial monolayers from unconjugated
986 BA-induced damage. Representative light microscopy images of H&E-stained Caco-2
987 monolayers after exposure to BA pools. Scale bar=20 μm. Unless otherwise specified, all
988 experiments were performed in triplicate. See Methods for statistical analyses. Data not marked
989 with asterisk(s) are not significant ****p<0.0001. Bars represent mean ± SEM.

990