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Identification of bile acid and fatty acid species as candidate rapidly bactericidal agents for topical treatment of gonorrhoea

Massachusetts Institute of Technology

1 **Identification of bile acid and fatty acid species as candidate rapidly bactericidal agents for**

2 **topical treatment of gonorrhea**

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22 Running title: Activity of bile acids and fatty acids against gonorrhea

23

24   **Synopsis**

25   Novel therapeutic strategies are urgently needed for *Neisseria gonorrhoeae*, given its increasing

26   antimicrobial resistance. Treatment of oropharyngeal *N. gonorrhoeae* infections has proven

27   particularly challenging, with most reported treatment failures of the first-line drug ceftriaxone

28   occurring at this site and lower cure rates in recent trials of new antibiotics reported for

29   oropharyngeal infections compared to other sites of infection. However, the accessibility of the

30   oropharynx to topical therapeutics provides an opportunity for intervention. Local delivery of a

31   therapeutic in a high concentration would enable the use of nontraditional antimicrobial

32   candidates, including biological molecules that exploit underlying chemical sensitivities of *N.*

33   *gonorrhoeae* but lack the potency or pharmacokinetic profiles required for effective systemic

34   administration.

35   **Methods**: Two classes of molecules that are thought to limit gonococcal viability *in vivo*, bile

36   acids and short- and medium-chain fatty acids, were examined for rapid bactericidal activity.

37   **Results**: The bile acids deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA), but not

38   other bile acid species, exerted extremely rapid bactericidal properties against *N. gonorrhoeae*,

39   reducing viability more than 100,000-fold after one minute. The short-chain fatty acids formic

40   acid and hexanoic acid shared this rapid bactericidal activity. All four molecules are effective

41   against a phylogenetically diverse panel of *N. gonorrhoeae* strains, including clinical isolates

42   with upregulated efflux pumps and resistance alleles to the most widely used classes of existing

43   antimicrobials. DCA and CDCA are both approved therapeutics for non-infectious indications

44   and are well-tolerated by cultured epithelial cells.

45   **Conclusions**: DCA and CDCA are attractive candidates for further development as anti-

46   gonococcal agents.

Introduction

Antimicrobial resistance (AMR) in *Neisseria gonorrhoeae* is increasing rapidly, threatening treatment efficacy as the incidence of gonorrhea also rises.[1] In the absence of an effective and widely-available vaccine, continued control of gonorrhea infections will require the development of novel therapeutic strategies that can surmount existing AMR.[2]

Most reported treatment failure of ceftriaxone, the current first-line treatment for gonorrhea, has occurred in oropharyngeal gonorrhea infections,[3-5] and success rates for antibiotics in recent clinical trials have been lowest for oropharyngeal compared to urogenital infections,[6-9] suggesting that this niche poses a particularly challenging environment for effective treatment.[10] The extent to which this is due to unfavorable pharmacokinetics is not known, but the evidence supports the oropharynx as a critical niche for the development of AMR in *N. gonorrhoeae*.[11]

In the pre-antibiotic era, topical antiseptics were a cornerstone of gonorrhea treatment. Interest in this strategy has resurged in the face of waning efficacy of antibacterial agents.[12] Prophylactic vaginal microbicides have been proposed,[13] although these have not thus far succeeded in clinical trials.[14] A topical antigonorrheal agent may be particularly suited to the challenge of clearing oropharyngeal gonorrhea, as it would circumvent pharmacokinetic challenges of targeting the oropharynx. Correctly applied, local treatment might also limit off-target toxicity both to the patient and to the microbiome, which in turn could reduce bystander selection for AMR in the flora.[15] The development of anti-infective mouthwashes as either a prophylactic or therapeutic tool to manage oropharyngeal gonorrhea infections has been of particular interest, with promising initial results showing reduction of viable *N. gonorrhoeae* loads in vitro and in patients [16, 17] but no efficacy shown in clinical trials.[18]

70   One underexplored advantage of topical administration is the ability to deliver

71   comparatively high concentrations of a drug to the site of infection, as the effective dose is not

72   limited by systemic steady-state concentrations. This property is particularly attractive, as it

73   reduces the potency required for an effective candidate therapeutic. As a result, almost any

74   unusual chemical sensitivities of *N. gonorrhoeae* to nontoxic compounds are theoretically

75   exploitable as a therapeutic strategy.

76   In *N. gonorrhoeae*, the MtrCDE efflux pump contributes to resistance to several

77   antibiotic classes, including macrolides and quinolones. In natural infection, MtrCDE effluxes

78   host-derived small hydrophobic molecules that are toxic to the bacterium. Important substrates of

79   this pump are thought to include fecal lipids [19] and bile salts [20-22] encountered during rectal

80   colonization. The fatty acid efflux pump FarAB may also be involved in fecal lipid efflux.[23]

81   The sensitivity of *N. gonorrhoeae* to these naturally occurring compounds presents a

82   therapeutic opportunity. The promise of fatty acids as a treatment strategy has been previously

83   noted, with particular emphasis on the sensitivity of *N. gonorrhoeae* to fatty acids with carbon

84   chain lengths of ten or more.[24-26] With the exception of initial investigations into a vaginal

85   hydrogel monocaprin prophylactic against HSV-2 in mice,[27] fatty acids have not been widely

86   explored as a treatment for sexually acquired gonorrhea infections, possibly because of their poor

87   solubility and unfavorable pharmacokinetic properties. However, a topically applied

88   monocaprin-based treatment for ophthalmia neonatorum is currently under development.[25, 26, 28,

89   29] The promise of monocaprin as a therapeutic suggests that other topical applications of fatty

90   acids – including for treatment of pharyngeal gonorrhea – are a rich route for further inquiry.

91   While the effects of medium- and long-chain fatty acids on the viability of *N. gonorrohoeae* have

92 been catalogued,[24, 25] there has not been a systematic investigation of short-chain fatty acids as

93 potential gonorrhea therapeutics.

94   The sensitivity of *N. gonorrhoeae* to different bile acid species has also not been

95 systematically explored, although one study reported inhibitory activity of several bile acid

96 derivatives against the laboratory strain MS11 and proposed adapting these into a prophylactic

97 vaginal microbicide.[30] Larger panels of bile acids have been tested for inhibition of growth of

98 other bacteria, such as *Helicobacter pylori*.[31] Testing a larger set of bile acids for antimicrobial

99 activity against *N. gonorrhoeae* may reveal additional candidates for novel topical therapeutics,

100 especially as some bile acid species are already approved drugs for other indications (e.g.,

101 ursodeoxycholic acid for primary biliary cirhossis; cholic acid for bile synthesis disorders;

102 chenodeoxycholic acid for gallstone dissolution; and injectable deoxycholic acid to reduce fat

103 below the chin).

104   In this work, we examine fatty acids with a range of chain lengths and a panel of bile

105 acids for rapid bactericidal activity. Two short-chain fatty acids, formic acid and hexanoic acid,

106 and two bile acids, deoxycholic acid and chenodeoxycholic acid, reduced viability of *N.*

107 *gonorrhoeae* to below the limit of detection (at least 100,000-fold) after one minute of exposure.

108 Each of these candidates was effective at rapidly killing a range of *N. gonorrhoeae* strains,

109 including clinical isolates with high-level resistance to first-line antibiotics and with hyperactive

110 efflux pump mutations. Further development of these new candidates may enable a topical

111 therapeutic strategy for oropharyngeal gonorrhoeae that is both rapidly effective and robust to

112 existing AMR in the *N. gonorrhoeae* population.

113

114 **Materials and methods**

115 **Bacterial strains and culture conditions.**

116   *N. gonorrhoeae* strains are presented in Table 1. All strains were cultured on GCB agar

117   (Difco) supplemented with 1% IsoVitaleX (Becton Dickinson) at 37°C with 5% CO_2.

118   **Bacterial killing assays.**

119   *N. gonorrhoeae* strains were grown overnight on GCB agar supplemented with 1%

120   IsoVitaleX then suspended in pre-warmed Graver-Wade medium. Bacterial suspensions were

121   mixed with each bile acid or fatty acid and incubated for 60 seconds at ambient temperature

122   without shaking, with a final bacterial concentration of OD_{600} 0.1. Exposure was halted by

123   immediate ten-fold dilution in Graver-Wade medium, and bacterial survival was assessed by

124   dilution plating on GCB agar supplemented with 1% IsoVitaleX. See Text S1 for additional

125   details.

126   All bacterial viability assays were performed at least twice; representative data from one

127   experiment is shown for each condition, except where repeated experiments yielded disparate

128   results (e.g. certain short-chain fatty acids; see Supplemental data file).

129   **Cell toxicity assays.** HEK 293T and Caco-2 cells were propagated in DMEM (ATCC® 30-

130   2002) supplemented with 10% FBS (ATCC® 30-2020) + 1X Penicillin-Streptomycin (Corning

131   30-002-CI). HeLa cells were propagated in RPMI-1640 Medium (ATCC® 30-2001)

132   supplemented with 10% FBS (ATCC® 30-2020) + 1X Penicillin-Streptomycin (Corning 30-002-

133   CI).

134   Cells in log phase growth were washed once with PBS without calcium and magnesium,

135   pH 7.4 (Corning 21-040-CM), then detached with 0.25% trypsin/2.21 mM EDTA (Corning 25-

136   053-CI) for 5-10 minutes and quenched in complete media. Cells were counted with an ORFLO

137   Moxi cell counter. 50,000 cells in 0.2 mL of complete media were seeded in each well of a 96

138   well tissue culture plate and incubated for 18-24 hours at 37°C with 5% CO_2 to allow for

139   adherence.

140   Following adherence, media was aspirated off and replaced with 0.2 mL media

141   supplemented with each compound or ethanol vehicle control at the specified concentration.

142   Cells were incubated at 37°C for 60 seconds. Exposure was halted by removing the

143   supplemented media and washing 3 times with 0.2 mL PBS. 0.2 mL MTT reagent in media (0.5

144   mg/mL) was added to each well. The contents were mixed and incubated at 37°C for 2 hours.

145   Absorbance at 540 nm (A_{540}) was measured on an Infinite® M1000Pro (Tecan) reader. Cells that

146   were subjected to media alone provided a baseline for viability at the time of assay, with percent

147   viability calculated as follows: [sample A_{540} - blank A_{540}]/[baseline A_{540} - blank A_{540}] × 100. Six

148   technical replicates were performed for each condition.

149

150   **Results**

151   **Deoxycholic acid and chenodeoxycholic acid exert rapid bactericidal activity against *N.***

152   ***gonorrhoeae*.**

153   Because some bile acids are approved drugs with favorable toxicity and safety profiles,

154   and because a topical application would allow us to deliver a high concentration to the target site,

155   we sought to determine whether bile acids could overwhelm the MtrCDE efflux pump and cause

156   bacterial killing.

157   Seven bile acids – cholic acid, deoxycholic acid, chenodeoxycholic acid,

158   urseodeoxycholic acid, glycocholic acid, lithocholic acid, and taurocholic acid – were assayed

159   for rapid bactericidal activity against the *N. gonorrhoeae* strain FA1090. Among this group, we

160   found two bile acids that were very effective at quickly killing *N. gonorrhoeae*: no cfus were

161   recovered after 60 seconds of exposure to either 1 mg/mL deoxycholic acid or chenodeoxycholic

162   acid, representing a minimum of 4-5 logs of killing (Figure 1).

163   In contrast to deoxycholic acid and chenodeoxycholic acid, none of the remaining bile

164   acids showed bacterial killing after 60 seconds of exposure at a concentration of 1 mg/mL

165   (Figure S1). This is particularly striking given extreme similarity of the chemical structures of

166   these bile acids. For example, no killing was observed in the presence of 1 mg/mL

167   ursodeoxycholic acid, a stereoisomer of chenodeoxycholic acid (Figure S1D).

168   Initial attempts to characterize the minimal bactericidal concentration via doubling

169   dilution found complete killing of FA1090 at 0.5 mg/mL for both deoxycholic acid and

170   chenodeoxycholic acid, with virtually complete survival of FA1090 at 0.25 mg/mL. Dose-

171   response experiments conducted at a finer scale showed a steep efficacy curve for both

172   deoxycholic acid and chenodeoxycholic acid, with partial bactericidal activity between 0.3

173   mg/mL and 0.4 mg/mL (0.76-1.0 mM) for each of these bile acids (Figure 2). The minimal

174   bactericidal concentration required to kill 99% of the bacterial population (MBC_{99}) was between

175   0.3 and 0.4 mg/mL for deoxycholic acid. The MBC_{99} of chenodeoxycholic acid was similar,

176   between 0.4 and 0.5 mg/mL.

177   **Formic acid and hexanoic acid exert rapid bactericidal activity against *N. gonorrhoeae*.**

178   The high sensitivity of *N. gonorrhoeae* to fatty acids has led to the routine inclusion of

179   soluble starch in gonococcal growth media to sequester contaminating fatty acids[32] and is also

180   the proposed explanation for the growth inhibition of *N. gonorrhoeae* by certain types of fecal

181   lipids.[19] We therefore sought to determine whether one or more fatty acids was sufficiently toxic

182   to *N. gonorrhoeae* to merit consideration as a therapeutic.

183   A panel of fatty acids with short and medium chain lengths were tested as above for rapid

184   bactericidal activity against the FA1090 strain. The panel comprised formic acid (C1:0), acetic

185   acid (C2:0), propionic acid (C3:0), butyric acid (C4:0), isobutyric acid (C4 branched chain),

186   valeric acid (C5:0), isovaleric acid (C5 branched chain), hexanoic acid (C6:0), octanoic acid

187   (C8:0), decanoic acid (C10:0), lauric acid (C12:0), palmitic acid (C16:0), oleic acid (C18:1), and

188   linoleic acid (C18:2). These were chosen to represent fatty acids of varying chain lengths, as well

189   as to cover the group of fecal lipids that has previously been postulated to prevent gonococcal

190   growth in fecal extracts (palmitic acid, oleic acid, and linoleic acid).[19]

191   No viable cfus were recovered after 60 seconds of incubation with 1% (v/v) formic acid

192   (C1:0) or hexanoic acid (C6:0) (Figure 3), although similar fatty acids such as acetic acid (C2:0),

193   proprionic acid (C3:0), isobutyric acid (C4), and octanoic acid (C8:0) failed to kill FA1090 at

194   this time point, as did longer chain fatty acids (Figure S2). Several short-chain fatty acids –

195   butyric acid (C4:0), valeric acid (C5:0), and isovaleric acid (C5) – showed promising

196   antibacterial activity but high variability between replicates (Figure S3), which may be a result of

197   incomplete mixing or partitioning of the fatty acid from the aqueous medium. This latter group

198   of short-chain fatty acids may include good candidates for further testing and optimization,

199   particularly combined with work to optimize solubility. As formic acid and hexanoic acid both

200   resulted in consistently high bactericidal activity, we focused on further characterizing these

201   compounds.

202   As with deoxycholic and chenodeoxycholic acid, dose-response experiments with formic

203   acid and hexanoic acid showed a sharp change in bactericidal activity, and doubling dilutions

204   were not sufficient to resolve the dose/response curve. Finer-scale dose-response experiments

205   showed partial bactericidal activity of formic acid at 0.3-0.4% (v/v) (MBC_{99} between 0.3% and

206   0.4%) (Figure 4A). Hexanoic acid was slightly less potent, with no bactericidal activity below

207   0.7% (v/v) (MBC$_{99}$ between 0.7% and 0.8%) (Figure 4B).

These compounds rapidly kill diverse clinical isolates of *N. gonorrhoeae*, including isolates

with antimicrobial resistance.

210   Some variants that provide resistance to existing classes of antimicrobial drugs – such as

211   mutations that impact the function and regulation of the MtrCDE efflux pump resulting in

212   increased macrolide resistance (e.g. [33-36]) – could also collaterally increase resistance to fatty

213   acids and bile salts. Because AMR in clinical *N. gonorrhoeae* populations is driving the need for

214   novel therapeutics, we tested the bile acid and fatty acid candidate compounds to determine their

215   efficacy against strains with AMR-associated alleles, including efflux pump variants.

216   Candidate compounds were tested against a diverse panel of *N. gonorrhoeae* laboratory

217   strains and clinical isolates. The strains selected include clinically relevant resistance alleles and

218   major variants in relevant efflux pumps, including overexpression and interspecies mosaic alleles

219   of the MtrCDE efflux pump, as well as promoter and loss-of-function variants in the fatty acid

220   efflux pump FarAB. These strains also included an isolate with reduced ceftriaxone

221   susceptibility conferred by the *penA* 60.001 allele from the internationally disseminated FC428

222   clone.[37] Table 1 describes the strains in this panel and their relevant characteristics.

223   Among the eight *N. gonorrhoeae* strains in this panel, we saw no variability in the

224   efficacy of rapid killing by any of the four candidate compounds: 60 seconds of exposure to 1

225   mg/mL deoxycholic acid, 1 mg/mL chenodeoxycholic acid, 1% (v/v) formic acid, or 1% (v/v)

226   hexanoic acid in GW media was sufficient to reduce the number of viable bacteria below

227   detectable levels in all cases (Figure 5). Resistance-associated mutations, including those thought

228 to increase MtrCDE efflux pump activity in MS11, GCGS0759, and GCGS0402, did not reduce

229 the efficacy of killing by either of the bile acids or the fatty acids tested.

230 **Epithelial cell toxicity of candidate compounds.**

231 To evaluate the potential of each candidate molecule as a therapeutic, three epithelial cell

232 lines were exposed to various concentrations of the candidate compounds for 60 seconds and cell

233 viability was assessed by MTT assay, with a goal of determining survival of cells at

234 concentrations at or above the MBC_{99} of each compound for FA1090.

235 Formic acid did not cause significant toxicity at 0.3% (v/v), but some epithelial cell lines

236 (Caco-2 and HEK239T) showed sensitivity to formic acid at concentrations above the MBC_{99} for

237 FA1090 (0.4% v/v and above). At 1% (v/v) formic acid, survival of all three epithelial cell lines

238 was marginal (Figure 6A). Hexanoic acid reduced viability to below 10% for all three cell lines

239 tested at a dose below the MBC_{99} for FA1090 (0.7% v/v) (Figure 6B). This agrees with

240 observations of poor viability of epidermal tissue in contact with undiluted formic or hexanoic

241 acid after a 3-minute exposure time.[38]

242 By contrast, epithelial cell viability was not compromised in the presence of up to 0.5

243 mg/mL deoxycholic acid or chenodeoxycholic acid, a concentration above the MBC_{99} of these

244 compounds for FA1090 (Figure 6C-D). Viability of Caco-2 cells in the presence of 0.3 mg/mL

245 deoxycholic acid was significantly (but likely spuriously) increased compared to the vehicle

246 control. Deoxycholic acid at 1 mg/mL resulted in a moderate but significant reduction in

247 viability for all three cell lines, and 1 mg/mL chenodeoxycholic modestly reduced viability of

248 HEK239T cells.

249

250 **Discussion**

251   Oropharyngeal gonorrhea is particularly difficult to treat, but relatively accessible to a

252   topical therapeutic. Here, we examined two host-derived classes of small molecules that have

253   been reported to interfere with the viability of *N. gonorrhoeae*. While these compounds may lack

254   the potency to succeed as traditional systemically administered antimicrobials, using a topical

255   formulation to deliver a locally high concentration of drug directly to the infection site could

256   harness their rapid bactericidal activity for therapeutic use.

257   Bile acids are thought to be an important physiological substrate of the MtrCDE efflux

258   pump.[19, 20] Several studies have demonstrated *in vitro* growth inhibition of *N. gonorrhoeae* by

259   cholic acid.[21, 22] The absence of a functional MtrCDE pump modestly increases growth inhibition

260   by cholic acid (roughly twofold decrease in MIC), as well as glycocholic acid, taurocholic acid,

261   and taurolithocholic acid.[30] We have expanded upon these observations by screening a panel of

262   bile salts for rapid bactericidal activity, and finding that two of them – deoxycholic acid and

263   chenodeoxycholic acid – have extremely rapid bactericidal activity, exerting at least five logs of

264   bacterial killing in a 60-second exposure window. This is the first evidence that bile salts could

265   be adapted as a rapidly effective topical therapeutic for gonorrhea.

266   Some of the most promising recent work in the field of topical gonorrhea treatment

267   revolves around the use of monocaprin, a 10-carbon fatty acid, as a treatment for neonatal eye

268   infection.[24-26, 29] Other medium- and long-chain fatty acids also have reported *in vitro*

269   antimicrobial activity against *N. gonorrhoeae* and are additional candidates for topical

270   treatment.[24, 25] We did not observe the large-magnitude bactericidal effects of some medium-

271   chain fatty acids, such as lauric acid, that have been previously reported.[24] This may stem from

272   differences in assay conditions, including buffer composition, drug concentration, and delivery

273   vehicle concentration. We observed partitioning of longer-chain fatty acids in our media, which

274 could account for variability between replicates. However, when we examined a panel of short-

275 chain fatty acids that may be suitable for use as an oropharyngeal therapeutic, we found that

276 formic acid and hexanoic acid are both reliably, rapidly bactericidal against *N. gonorrhoeae*.

277 These compounds have not previously been reported as candidate agents for treatment of *N.*

278 *gonorrhoeae* infection; however, the potential toxicity of these compounds may require

279 alternative formulations. Optimizing delivery strategies and drug formulations can also minimize

280 undesirable side effects, as in the case of a candidate bile acid therapeutic for irritable bowel

281 syndrome.[39] This will be a critical future step in the development of these candidates for clinical

282 use as anti-infectives.

283   Deoxycholic acid, chenodeoxycholic acid, formic acid, and hexanoic acid are all rapidly

284 bactericidal against diverse strains of *N. gonorrhoeae*, including those with increased MtrCDE

285 expression and high-level resistance to the antibiotics in clinical use (ceftriaxone, azithromycin,

286 and ciprofloxacin; Table 1). It is possible that some variants represented in our strain panel might

287 result in increased susceptibility to some of these compounds. For example, as FarAB effluxes

288 fatty acids, the *farA* loss-of-function mutation in the GCGS0449 strain may increase

289 susceptibility to formic acid, hexanoic acid, or both. As our focus was to determine whether

290 common resistance alleles confer resistance to the candidate compounds, we did not test whether

291 these variants reduce the MBC or increase the kinetics of bacterial death.

292   For each of these four candidate molecules, the mechanism of *N. gonorrhoeae* killing is

293 not known. Hexanoic acid may act via a detergent-like disruption of cell membranes, which

294 would also explain its high toxicity against epithelial cell lines. Bile salts also have detergent-like

295 properties, but the specificity of the rapid bactericidal effect we observe – even stereospecificity,

296 in the case of ursodeoxycholic acid versus chenodeoxycholic acid – suggests a more specific

297 mechanism of bacterial killing. Interestingly, similar stereospecificity was observed in the

298 growth inhibition of some *Helicobacter pylori* strains by chenodeoxycholic acid, but not

299 ursodeoxycholic acid, suggesting a conserved biological distinction between these

300 stereoisomers.[40] Future work defining the mechanism of action for each of these compounds

301 against *N. gonorrhoeae* will also be crucial in defining bacterial pathways to resistance, which in

302 turn will allow us to estimate how easily resistance may arise in a clinical setting and whether

303 implementation of fatty acid or bile acid-based therapeutics might select for collateral resistance

304 against other types of drugs. For example, some bile acids, including chenodeoxycholic acid,

305 transcriptionally derepress the MtrCDE pump.[41]

306   Given the need for novel antibiotics and treatment strategies, the candidate therapeutics

307 we describe here are advantageous in many ways. First, a topical administration route such as we

308 propose will allow us to directly target high concentrations of bactericidal compounds to the

309 oropharynx. Second, by focusing on classes of compounds that *N. gonorrhoeae* is unusually

310 sensitive to, we may be able to limit off-target effects on the normal flora, which would also help

311 reduce bystander selection. This effect is compounded with a topical administration approach,

312 which will limit exposure of the microbiome in other compartments, particularly in comparison

313 to standard systemic therapy. Third, the extremely rapid bactericidal kinetics we describe here

314 are well-suited to the challenge of treating gonorrhea in the setting of sexual health clinics, where

315 the simplicity of point-of-care single-dose treatment regimens (e.g., ceftriaxone) provides

316 significant advantages. Fourth, exploring the use of small biological compounds rather than

317 novel chemical libraries enables us to take advantage of preexisting safety data and (in the case

318 of some bile acids) approval for use in other clinical contexts to streamline the process of moving

319 compounds from preclinical investigation to clinical trials. This is particularly important because

320 there is no established animal model for pharyngeal gonorrhea. However, in the case of

321 candidates that are already approved therapeutics for other indications – such as deoxycholic

322 acid and chenodeoxycholic acid – supplementing existing safety data with formulation-specific

323 toxicity data in animals may be sufficient to permit direct progression to human trials.

324   The candidate compounds we describe here are well-suited to this paradigm: rapidly

325 effective against diverse *N. gonorrhoeae* strains, including clinical isolates with substantial AMR

326 phenotypes, and ripe for development into topical formulations.

338 **Transparency declarations**

339 The authors declare that the findings presented in this manuscript are being submitted as a

340 provisional patent application. YHG has consulted for GSK and Quidel, serves on the scientific

341 advisory board of DayZeroDiagnostics, and has received research support from Pfizer and

342 Merck.

343   Complete details of all relationships for profit and not for profit for GT can be found at the

344   following link: https://www.dropbox.com/sh/szi7vnr4a2ajb56/AABs5N5i0q9AfT1IqIJAE-

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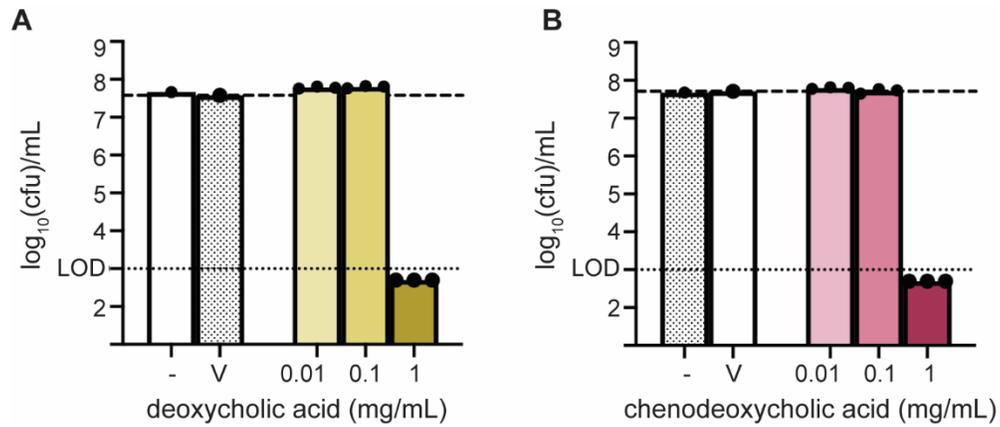
Figure 1. Rapid killing of *N. gonorrhoeae* FA1090 by deoxycholic acid and chenodeoxycholic acid. Survival of FA1090 in Graver-Wade (GW) medium with no supplementation (-), GW co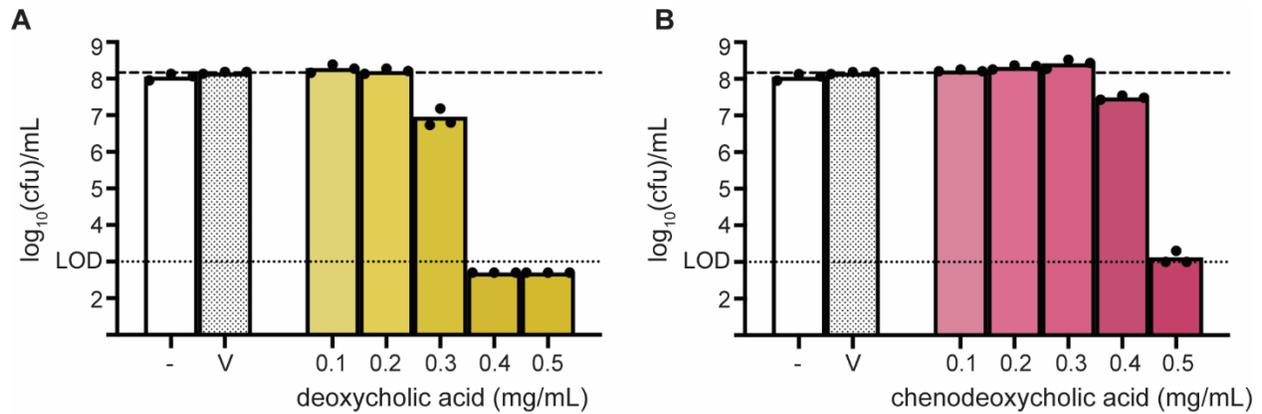ntaining 2% ethanol (vehicle control, V), or GW with varying concentrations of (**A**) deoxycholic acid or (**B**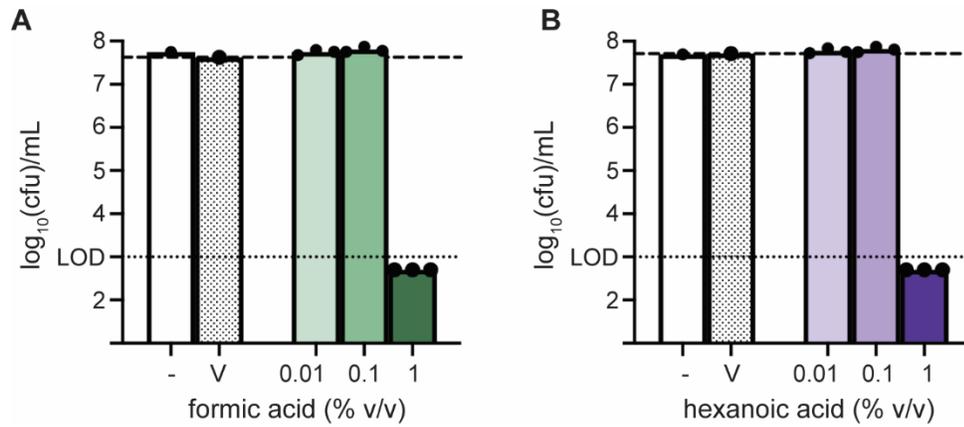) chenodeoxycholic acid. Samples were mixed gently and incubated at ambient temperature for one minute, after which exposure was halted by dilution and plating. Dotted line indicates the limit of detection (LOD, 10^3 cfu/mL). Dashed line indicates bacterial survival in the vehicle control condition.

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Figure 2. Dose-dependent killing of FA1090 by deoxycholic acid and chenodeoxycholic acid. Survival of FA1090 in Graver-Wade (GW) medium with no supplementation (-), GW containing 1% ethanol (vehicle control, V), or GW with v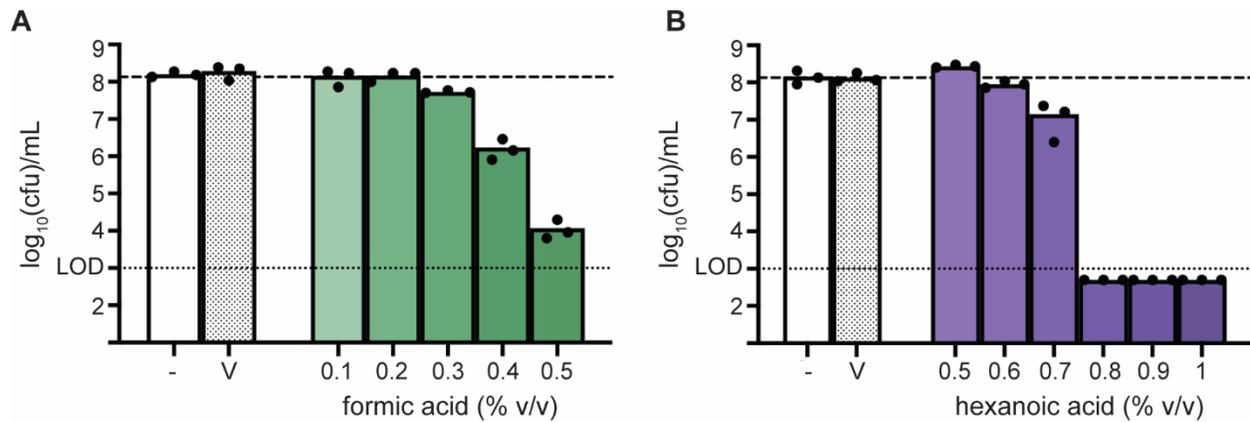arying concentrations of (**A**) deoxycholic acid or (**B**) chenodeoxycholic acid. Samples were mixed gently and incubated at ambient temperature for one minute, after which exposure was halted by dilution and plating. Dotted line indicates the limit of detection (LOD, 10^3 cfu/mL). Dashed line indicates bacterial survival in the vehicle control condition. Control groups are shown in both panels for ease of reference.

Figure 3. Rapid killing of *N. gonorrhoeae* FA1090 by formic acid and hexanoic acid.

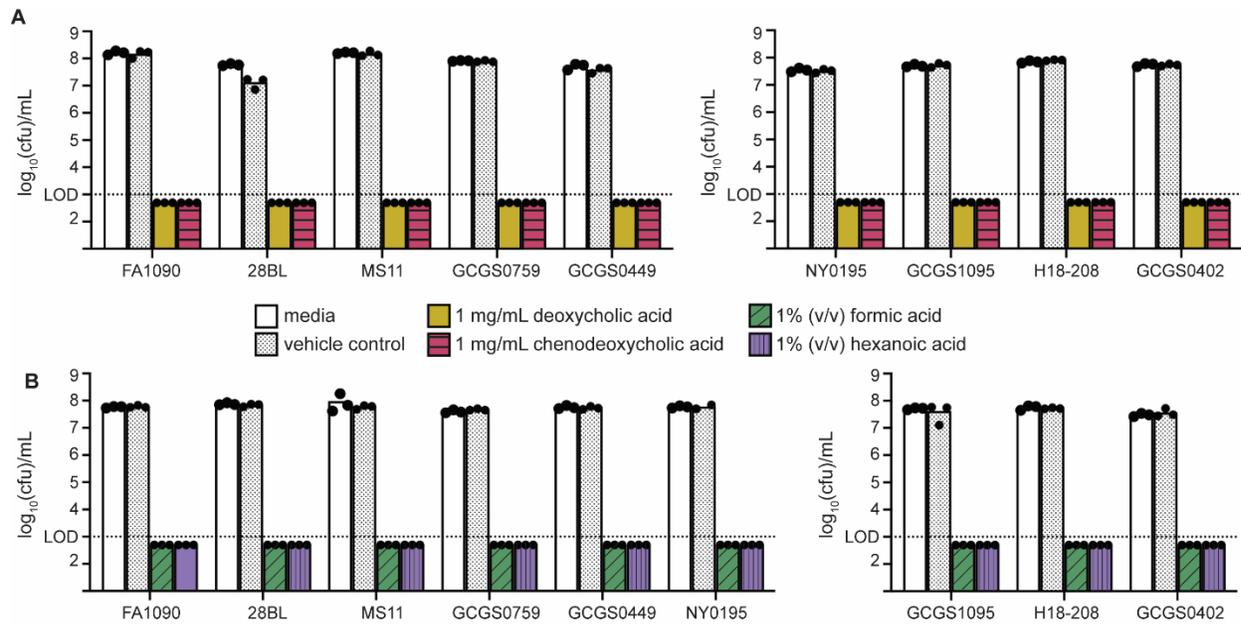Survival of FA1090 in Graver-Wade (GW) medium with no supplementation (-), GW containing

1% ethanol (vehicle control, V), or GW with varying concentrations of (**A**) formic acid or (**B**)

hexanoic acid. Samples were mixed gently and incubated at ambient temperature for one minute,

after which exposure was halted by dilution and plating. Dotted line indicates the limit of

detection (LOD, 10^3 cfu/mL). Dashed line indicates bacterial survival in the vehicle control

condition.

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Figure 4. Dose-dependent killing of FA1090 by formic acid and hexanoic acid. Survival of

FA1090 in Graver-Wade (GW) medium with no supplementation (-), GW containing 1% ethanol

(vehicle control, V), or GW with varying concentrations of (**A**) formic acid or (**B**) hexanoic acid.

Samples were mixed gently and incubated at ambient temperature for one minute, after which

exposure was halted by dilution and plating. Dotted line indicates the limit of detection (LOD,

10^3 cfu/mL). Dashed line indicates bacterial survival in the vehicle control condition.

Figure 5. Rapid killing of diverse *N. gonorrhoeae* strains by deoxycholic acid, chenodeoxycholic acid, formic acid, and hexanoic acid. Survival of various strains in Graver-Wade (GW) medium without supplementation (-), GW containing ethanol (vehicle, V), or GW with (**A**) 1 mg/mL deoxycholic acid (yellow) or 1 mg/mL chenodeoxycholic acid (red) (vehicle control, 2% ethanol), or (**B**) 1% (v/v) formic acid (green) or 1% (v/v) hexanoic acid (purple) (vehicle control, 1% ethanol). Samples were mixed gently and incubated at ambient temperature for one minute, after which exposure was halted by dilution and plating. Dotted line indicates the limit of detection (LOD, 10^3 cfu/mL).
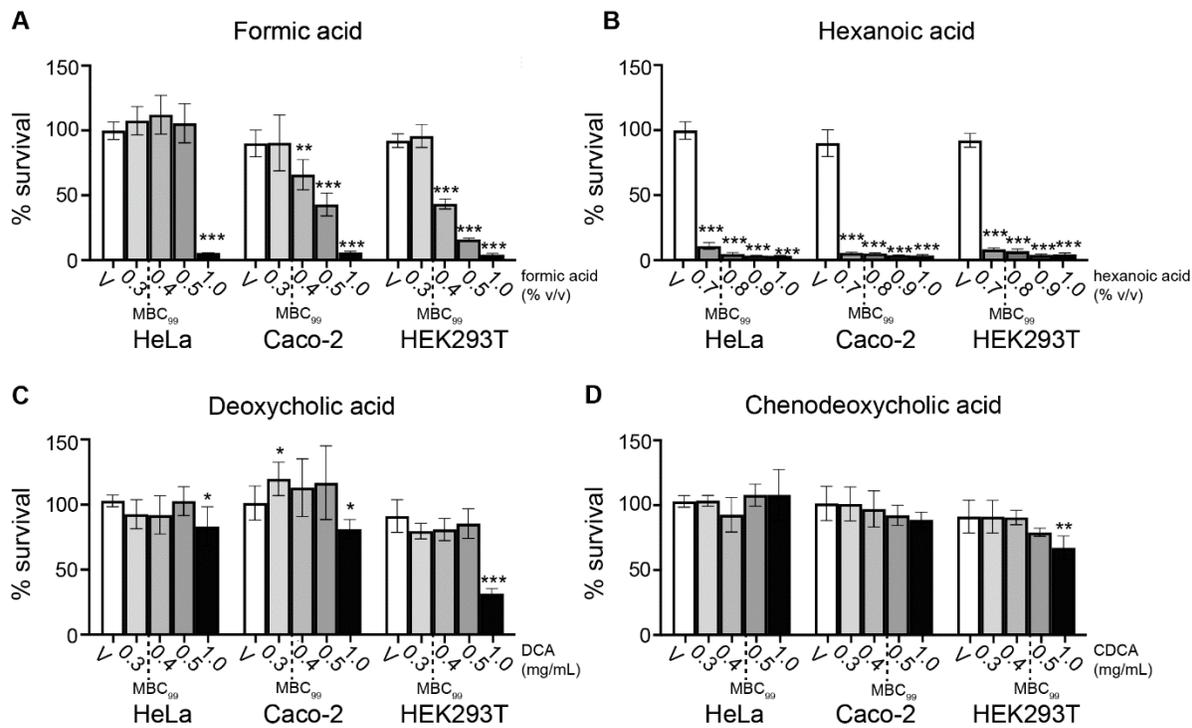
Figure 6. Epithelial cell toxicity of candidate compounds. Survival of 5×10^4 adherent HeLa,

Caco-2, or HEK293T cells exposed media supplemented with ethanol (vehicle, V) at 1%

(panels A-B) or 2% (panels C-D), or with various concentrations of (**A**) formic acid, (**B**)

hexanoic acid, (**C**) deoxycholic acid, or (**D**) chenodeoxycholic acid. After addition of each

compound or vehicle control, cells were incubated at ambient temperature for one minute, after

which the media was removed and exposure was halted by washing three times with PBS. Cell

viability was evaluated by MTT assay. Shown: mean and standard deviation of 6 technical

replicates, normalized to cell viability with no chemical or vehicle exposure (media only).

Statistical significance was tested for each condition against the appropriate vehicle control by

2-tailed t test with Bonferroni correction; * $p<0.05$; ** $p<0.01$; *** $p<0.001$. MBC_{99} for each

compound against FA1090 is shown on the x-axis for reference.

533

534 **Table 1. *N. gonorrhoeae* strains and relevant characteristics.** The susceptibility of each strain

535 to ciprofloxacin (CIP), azithromycin (AZI), and ceftriaxone (CRO) is presented, in addition to

536 genotypic characteristics relevant to antimicrobial susceptibility and efflux pump activity.

537 Susceptibility breakpoints were defined as follows: CIP^R, MIC > 1 μg/mL; AZI^R, MIC > 2

538 μg/mL; low-level CRO^{RS}, MIC 0.0625 μg/mL; CRO^{RS}, MIC ≥ 0.125 μg/mL. GCGS0402,

539 GCGS0449, GCGS0759, and GCGS1095 were collected by the Centers for Disease Control and

540 Prevention's Gonococcal Isolate Surveillance Project.[42] NY0195 was collected by the New York

541 City Public Health Laboratory, Department of Health and Mental Hygeine.[43] Sources for all

542 strains can be found in Text S1.

543

Strain	Antibiotic susceptibility	Relevant characteristics
FA1090	CIP^S, AZI^S, CRO^S	
28BL	CIP^S, AZI^S, CRO^S	*farA* promoter variants of unknown significance: single T insertion and G>A substitution
MS11	CIP^S, AZI^S, CRO^S	mtr_{120} promoter mutation [44]
H18-208	CIP^R, AZI^S, CRO^{RS}	Reduced cephalosporin susceptibility from *penA* 60.001 [37]
GCGS1095	CIP^S, AZI^S, CRO^{RS}	Reduced cephalosporin susceptibility from $RpoB^{R201H}$ [45]
GCGS0402	CIP^S, AZI^R, CRO^S	Macrolide resistance from *N. meningitides*-type mosaic *mtrCDE* allele [35]
GCGS0449	CIP^S, AZI^S, CRO^S	*farA* frameshift mutation (predicted FarAB loss-of-function)
GCGS0759	CIP^R, AZI^R, low-level CRO^{RS}	Reduced cephalosporin susceptibility from *penA* XXXIV;[46, 47] fluoroquinolone resistance from GyrA-91F/95G ParC-87R;[42] increased MtrCDE expression from single A nucleotide deletion in 13-bp repeat of *mtrR* promoter [48] likely contributing to macrolide and cephalosporin resistance; near phylogenetic neighbor of NY0195

| NY0195 | CIP^R, AZI^S, CRO^S | Fluoroquinolone resistance from GyrA-91F/95G ParC-87R;[42] *mtrC* frameshift mutation (MtrCDE loss-of-function) likely contributing to phenotypic macrolide and cephalosporin susceptibility; *penA* XXXIV (known to confer reduced cephalosporin susceptibility);[46, 47] single A nucleotide deletion in 13-bp repeat of *mtrR* promoter (known to increase MtrCDE expression);[48] near phylogenetic neighbor of GCGS0759 |

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