Niclosamide reverses SARS-CoV-2 control of lipophagy

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SUMMARY

The global effort to combat COVID-19 rapidly produced a shortlist of approved drugs with antiviral activities for clinical repurposing. However, the jump to clinical testing was lethal in some cases as a full understanding of the mechanism of antiviral activity as opposed to pleiotropic activity/toxicity for these drugs was lacking. Through parallel lipidomic and transcriptomic analyses we observed massive reorganization of lipid profiles of infected Vero E6 cells, especially plasmalogens that correlated with increased levels of virus replication. Niclosamide (NIC), a poorly soluble anti-helminth drug identified for repurposed treatment of COVID-19, reduced the

9 total lipid profile that would otherwise amplify during virus infection. NIC treatment reduced the

10 abundance of plasmalogens, diacylglycerides, and ceramides, which are required for virus

11 production. Future screens of approved drugs may identify more druggable compounds than

12 NIC that can safely but effectively counter SARS-CoV-2 subversion of lipid metabolism thereby

13 reducing virus replication. However, these data support the consideration of niclosamide as a

14 potential COVID-19 therapeutic given its modulation of lipophagy leading to the reduction of

15 virus egress and the subsequent regulation of key lipid mediators of pathological inflammation.

16

17 **Keywords:** lipidomics, autophagy, lipophagy, SARS-CoV-2, coronavirus, COVID-19, mass

18 spectrometry, RNA sequencing, niclosamide, antivirals, metabolism, chemical biology

19

20 **INTRODUCTION**

21   The current pandemic spread of SARS-CoV-2 has resulted in >600,000 deaths in the

22 United States alone and >176M cases and >3.8M deaths worldwide (Dong et al., 2020). In

23 2020, there was a rapid global effort to study severe acute respiratory syndrome coronavirus 2

24 (SARS-CoV-2) infection kinetics *in vitro* to identify pathways that are involved in entry, replica-

25 tion, and egress of the virus as new targets that can be treated with existing drugs (Gautret et

26 al., 2020; Jeon et al., 2020; Liu et al., 2020; Vincent et al., 2005; Wang et al., 2020; Zhou et al.,

27 2020). However, this worldwide effort was fraught with inconsistencies ranging from the mam-

28 malian host cell used for infection studies, to overall study design, which made it difficult to

29 identify common host cell pathways critical to virus biology that can be targeted with available

30 compounds.

31   Chloroquine (CQ) and, hydroxychloroquine (HCQ), which are indicated for treating ma-

32 laria, have been shown to also have antiviral activity against SARS-CoV-2 (D'Alessandro et al.,

33 2020; Dyall et al., 2014; Liu et al., 2020; Mauthe et al., 2018; Vincent et al., 2005; Wang et al.,

34 2020). However, the direct and rapid translation of this finding to clinical studies during the

35 pandemic was either equivocal at best, or disastrous (Axfors et al., 2021). Although no studies

36 to date have specifically and comprehensively explored the underlying antiviral mechanism of

37 action (MOA) for these two drugs, they nonetheless moved quickly to COVID-19 clinical stud-

38 ies. CQ and HCQ are 4-aminoquinolines (4-AQ), and another human-safe 4-AQ drug,

39 Amodiaquine (AQ), has also been shown to have antiviral activity against SARS-CoV-1 and

40 Middle East respiratory syndrome coronavirus (MERS-CoV) (Dyall et al., 2014; Vincent et al.,

41 2005), as well as Ebola (D'Alessandro et al., 2020), suggesting that there are common MOAs

42 underpinning broad 4-AQ antiviral activity. Initially, these 4-AQ compounds were considered as

43 candidate partner drugs that can be used along with remdesivir (Wang et al., 2020), the lead-

44 ing broad-spectrum RNA synthesis inhibitor that has shown some clinical benefit (Goldman et

45 al., 2021). The exact nature of the cellular pathways and processes that are targeted by these

46 4-AQs resulting in reductions in virus propagation remain unknown, but direct protein interac-

47 tion studies with CQ suggest broad pleiotropic effects on the cell (Gordon et al., 2020). The

48 pleiotropic effects of drugs such as CQ should have given pause, preventing its quick transla-

49 tion to the clinic wherein higher doses of this drug exacerbate the pleiotropic side effects that

50 can result in direct harm to the subject in clinical trials (Borba et al., 2020).

51   Another anti-parasitic drug, niclosamide (NIC), which suppresses MERS-CoV propaga-

52 tion by inhibiting autophagosome-lysosome fusion through the Beclin1 (Bec1, autophagy regu-

53 lator/antiapoptotic protein)-SKP2 (S-phase kinase-associated protein 2) pathway (Gassen et

54 al., 2019) has also been proposed to be a potent candidate drug for repurposing in the treat-

55 ment of SARS-CoV-2 (Xu et al., 2020). NIC is a salicylanilide, oral anti-helminthic drug used in

56　human and veterinary medicine. Akin to the 4-AQs, it appears to have functional effects be-

57　yond the Bec1-SKP2 axis in a cell, resulting in broad anti-infective properties, and as a result,

58　also suffers from poor translational potential to the clinic for COVID-19. Considering the failure

59　of CQ and HCQ to progress towards clinical utility, the candidacy of NIC, which is at an earlier

60　stage of consideration for COVID-19 treatment, compelled us to examine more closely its

61　global effects on host cells to determine if we can uncouple the reported antiviral MOA via the

62　Bec1-SKP2 pathway as well as other putative antiviral mechanisms from intrinsic cytotoxicity.

63　We hypothesize that NIC exerts pleiotropic functional activities on a host cell that result in the

64　perturbation of two intimately associated cellular pathways: autophagy and lipid metabolism.

65　These two processes are dysregulated at critical SARS-CoV-2 life cycle checkpoints during virus

66　infection (Stukalov et al., 2021), and treatment with NIC is hypothesized to reverse this

67　dysregulation. Recently, a multi-omics analysis of clinical samples from COVID-19 patients also

68　described a marked change in lipidomics profiles that correlated with disease severity (Overmyer

69　et al., 2021), but this approach could not characterize the mechanism at the cellular level. Here-

70　in, we identified and characterized autophagic or lipophagic pathways (and wider linked cellular

71　networks) that are targeted by NIC in the presence and absence of a SARS-CoV-2 infection.

72　We used the lipid signatures induced by this anti-parasitic compound to detect pathways that

73　are predicted to either lead to direct antiviral effects or to cellular dysregulation and cell death.

74　We discuss the utility of this approach in informing the selection of repurposed drugs that may

75　have more clinical benefit, better bioavailability, and lesser cytotoxicity in the context of

76　COVID-19 and other infectious diseases.

77

78　**RESULTS**

SARS-CoV-2 infection alters the host cell lipidome

79

80   The mechanism by which SARS-CoV-2 enters host cells and systematically alters the

81 cellular environment is currently one of the most compelling areas of study to support the de-

82 velopment of antiviral interventions (V'kovski et al., 2020). Evidence suggests that SARS-CoV-

83 2 subverts pre-existing cellular lipids and lipid signaling mechanisms for entry, intracellular traf-

84 ficking and egress (Abu-Farha et al., 2020). This is consistent with what is known about virus-

85 es in general (Mazzon and Mercer, 2014). To investigate the role of lipids in SARS-CoV-2 dur-

86 ing the infection cycle in host cells, we profiled the global lipidome from Vero E6 cells at 16h

87 and 48h post-infection with SARS-CoV-2 (**Figure S1A**). The correlation matrix across the

88 groups studied showed a distinct clustering in SARS-CoV-2 infected cells as well as clear clus-

89 tering with virus and NIC treatment (**Figure S1B**). In addition, a principal component analysis

90 (PCA) revealed clear clustering based on lipid profiles between early (16h) and late (48h)

91 timepoints post viral infection (**Figure S1C).** PCAs also showed clustering with NIC treatment

92 of virus infected cells at early (16h) and late timepoints (48h) (**Figures S1D and E, respec-**

93 **tively**). Measurement of supernatant SARS-CoV-2 genome copy with RT-qPCR revealed a

94 significantly higher quantity of released progeny genomes at 48h than 16h, indicating that

95 more viral egress is occurring at the 48h timepoint, which is consistent with literature **(Figure**

96 **S2)**. We did not perform any measurements of infectious progeny virions (i.e., plaque assay),

97 so the antiviral effect of NIC in this experiment cannot be conclusively determined.

98   As SARS-CoV-2 infection caused perturbations in the global lipid profiles (**Figure 2A**),

99 we next sought to characterize the modulations of different lipid classes between early (16h) vs

100 late (48h) timepoints post viral infection. In total, we identified 720 lipids from 34 classes (**Ta-**

101 **ble S2, S3**) in SARS-CoV-2 infected host cells covering all major lipid classes (**Figure 2A**).

102   Phosphatidylcholine (PC), phosphatidylethanolamine (PE), plasmalogens (ether linked lipids)

103   and triacylglycerol (TG) represented the most frequent lipid classes identified, which is ex-

104   pected given their abundance in cell membranes and lipid droplets (**Figure 2A, Figure 3**).

105   Hierarchical clustering analysis of the differentially regulated lipids (FC>1.5, adjusted p-

106   value <0.05) revealed specific lipid classes were associated with early or late events during

107   productive virus infection (**Figure 2A, Table S2**). We found that ether lipids including

108   plasmanyl-TG, plasmenyl-TG, plasmanyl-LPC, plasmanyl-PC, plasmenyl-PC, and plasmenyl-

109   PE, as well as diacylglycerides were elevated at 48h post viral infection (**Figure 2B, Table**

110   **S3**). We measured the relative abundance of the total ether linked lipids and observed a highly

111   significant elevation (P-value = 2.94E-38) of total ether linked lipids at 48h post viral infection

112   (**Figure 2C, Figure S3**), suggesting a potential role of ether linked lipids in SARS-CoV-2 path-

113   ogenesis. A comparative extracted ion chromatogram of plasmanyl-PC (O-18:0/16:0)+H, as an

114   example of a specific ether lipid is shown (**Figure 2D**) to convey the clear difference in profiles

115   between the two time points tested. On the other hand, TGs, cholesterol esters (CE), and

116   hexosylceramides (HexCer) were elevated at early stages of infection (16h) (**Figure 2A and B,**

117   **Figure 3, Figure S3, Table S3**). The repertoire of lipid classes that partitioned to early steps in

118   virus infection (16h) and later stages of increased virus replication and egress (48h) are shown

119   in **Figure 3**.

120   Lipids that contained longer chain fatty acids with higher degrees of unsaturation includ-

121   ing TGs (4-9 double bonds (db)), PC (4-5 db), CL (5-6 db), PE (1-6 db), and PS (1-4 db) were

122   elevated in early (16h) viral infection (**Figure 3, Table S3**), a trend consistent with what was

123   described in SARS-CoV-2 infected golden Syrian hamsters (Rizvi et al., 2021). In contrast,

124   saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and long chain fatty acids

125   (LCFA) were found to be significantly downregulated in early (16h) viral infection but elevated

126   at 48h post viral infection (**Table S3**), consistent with previous reports (Koyuncu et al., 2013;

127   Nguyen et al., 2018). We identified changes in lipids that may be related to a cellular response

128   to compensate for virus energy utilization, viral particle formation from lipid droplets, vesicle

129   transport and autophagosome formation, noting a significant increase in plasmalogens (ether

130   lipids), sphingolipids (SM and CerNS), glycerophospholipids (PI, PS and PG),

131   lysophospholipids (LPE) and glycerolipids (DG) and a significant decrease in TG and CE lipids

132   at 48h (**Figure 2A-B, Figure 3**).

133   We observed a differential regulation of lipids during increased levels of viral replication.

134   Of note, we observed significant increases in PG, PS and PI, but not a significant change in

135   PC or PE phospholipids. This differential regulation of phospholipids could suggest formation

136   of membranes for the viral envelope as most mammalian cells are high in PC and PE phos-

137   pholipids (Deng and Angelova, 2021). The significant decrease in TGs and CEs is likely relat-

138   ed to virus utilization of lipid droplets to make viral particles needed for replication. Lipid drop-

139   lets are used by cells to store neutral lipids that are utilized for energy needs, but viruses re-

140   quire these lipids for replication and thus hijack the lipid droplets to enable their own growth.

141   The increase in plasmalogens is also associated with viral replication and increased levels

142   have been detected in the serum of patients with ZIKV and other viruses (Cloherty et al., 2020;

143   Mazzon and Mercer, 2014).

144

145   **Niclosamide treatment distinctly impacted ether lipids and this effect correlated with in-**

146   **creased viral replication and virus egress**

147   To understand these lipid modulations in the context of therapeutic potential, we used

148   niclosamide (NIC), an anti-parasitic drug, which was found to suppress MERS-CoV propaga-

149   tion by enhancing autophagic flux through the Beclin1 (autophagy regulator/antiapoptotic pro-

150   tein)-SKP2 (S-phase kinase-associated protein 2) pathway (Gassen et al., 2019). NIC has also

151   been proposed to be a potent candidate drug for repurposing in the treatment of SARS and

152   COVID-19 (Gassen et al., 2021; Pindiprolu and Pindiprolu, 2020; Wu et al., 2004). The influ-

153   ence of SARS-CoV-2 infection on Vero E6 cell lipid metabolism, and inhibition of autophagic

154   flux encouraged us to assess if NIC may impart therapeutic potential to SARS-CoV-2 infection.

155   We first examined the effect of NIC on Vero E6 cells in the absence of infection to es-

156   tablish a baseline for both the 16h and 48h time points (**Figure 4, Figure S4, Table S7)**. In to-

157   tal, we identified 520 lipids across all samples, with distinct profiles between DMSO control and

158   NIC treated cells. We observed a profound reduction across both time points in the lipid pro-

159   files for TG, and this effect was more pronounced at the 48h time point (**Figure 4A**). In the ab-

160   sence of NIC treatment, plasmalogens (plasmanyl-PE, -TG, and -PC as well as plasmenyl-

161   LPC, -PE, -LPE, -PC and -TG) were found to be differentially abundant at 48h as compared to

162   16h, which is expected given the cell growth during this time frame (**Figure 4A and Table S4**).

163   However, at the 48h time point (24h post treatment) NIC reduced the abundance (>2 log2 fold-

164   change) of HexCer-NDS, while in parallel, increased the abundance (>2 log2 fold-change) of

165   plasmenyl- and plasmanyl-TG, PI, plasmanyl-PE and -PC, as well as BMPs (**Figure 4B and**

166   **Figure S5**).

167   Having established a baseline effect of NIC on lipid metabolism in Vero E6 cells, we

168   next sought to understand the effect of NIC on lipid metabolism during SARS-CoV-2 infection.

169   We globally profiled lipids from cells infected with SARS-CoV-2 and treated with DMSO as

170 compared to cells infected with SARS-CoV-2 and treated with NIC at 16h (**Figure S1B**) and

171 48h (**Figure 5A, Figure S1E**) using our UHPLC-HRMS approach. A PCA identified a clear

172 separation between NIC treated and untreated SARS-CoV-2 infected samples (**Figure S1B, D**

173 **and E**). We observed that the treatment of Vero E6 cells with NIC for 24h starting 24h after

174 SARS-CoV-2 infection (48h) robustly impacted the global lipid profile as compared to NIC

175 treatment beginning simultaneously with SARS-CoV-2 infection (**Figure 5B**), suggesting that

176 the antiviral activity of NIC is also dependent on host cell state (in this case, infection as op-

177 posed to cell growth described above), which in turn correlated with higher levels of virus repli-

178 cation and virion production (**Figure S2**). However, no significant reduction in viral genome

179 copy was observed in (NIC) treated cells compared to DMSO controls at either timepoint (**Fig-**

180 **ure S2**). This corroborates previous findings that NIC does not act to inhibit virus entry or ge-

181 nome replication (Gassen et al., 2021; Pindiprolu and Pindiprolu, 2020). NIC reduced DG as

182 well as several plasmalogens, both of which would have otherwise been increased by SARS-

183 CoV-2 infection at the 48h time point (**Figure 5A**, **Figure 2, Figure 3**). Strikingly, we observed

184 that NIC treatment downregulated total ether lipids (P= 2.49E-38) (**Figure 5C**), suggesting that

185 NIC may impart anti-viral effect via affecting ether lipid metabolism. As indicated earlier, we

186 observed an increase in ether lipids at 48h in cells infected with virus alone, suggesting a key

187 role of ether lipids in viral replication **(Figure 2C)**. Upon treatment with NIC, it appears that this

188 lipid pathway is disrupted significantly, and thus may be affecting viral replication.

189

190 **Transcriptional profiling captures the broader cellular impact of virus infection and**

191 **treatment with niclosamide**

192   We assessed if virus-induced changes in lipidomic profiles corresponded to canonical

193   transcriptional regulation of genes that are known to be involved in lipid metabolism, autopha-

194   gy, phosphorylation, and vesicle transport. Furthermore, we explored if treatment with NIC al-

195   ters this profile, and whether time of NIC addition alters the course of infection. To do this, we

196   used RNASeq to capture the global cellular gene expression of i) Vero E6 cells over time (ef-

197   fect of time), ii) Vero E6 cells during cellular SARS-CoV-2 infection (effect of virus) and iii) fol-

198   lowing treatment with NIC (effect of drug).

199   We did not observe any significantly differentially expressed (DE) genes over time in our

200   Vero E6 culture alone (0h vs 16h, 0h vs 48h, 16h vs 48h) (**Table S6**), suggesting while the cel-

201   lular background of Vero E6 cells during these different time periods may be dynamic, the age

202   of the cell culture did not confound other comparisons made in this study. Similarly, we did not

203   observe any differences between cells treated with SARS-CoV-2 and NIC at 16h versus 48h.

204   We did, however, observe differences with NIC treated cells at 16h versus 48h (860 DE genes,

205   474 up-regulated and 386 down-regulated at 48h) (**Table S1B, Table S6**), as well as SARS-

206   CoV-2 infected cells at 16h versus 48h (109 DE genes, 48 up-regulated and 61 down-

207   regulated at 48h) (**Table S1B, Table S6**).

208   SARS-CoV-2 infection resulted in significantly DE genes across all our comparison

209   groups (**Table S1B, Table S5, Figure 6**. Viral infection resulted in 585 DE genes at 16h (474

210   up-regulated; 111 down-regulated), and 562 DE genes at 48h (433 up-regulated; 129 down-

211   regulated) (**Table S5**). SARS-CoV-2 and NIC conditions resulted in more DE genes than

212   SARS-CoV-2 infection alone; NIC and viral infection resulted in 3,083 DE genes (1,301 up-

213   regulated; 1,782 down-regulated) at 16h, and 1,595 DE genes (706 up-regulated, 889 down-

214   regulated) at 48h (**Table S5**). Overrepresented GO terms associated in both up- and down-

215   regulated DE datasets contained a large number of lipid phosphate metabolism, lipid transport,

216   autophagy and lysosome-associated terms. Terms related to phosphatidylinositol,

217   phosphatidylethanolamine and phosphatidylglycerol were especially prevalent, mirroring ob-

218   served changes in the lipidomics data. At 16h, viral infection induced an up-regulation of

219   GABARAPL1, MAP1LC3A, PIK3C3, USP30-ORG3a and TBK1 (also up-regulated at 48h), and

220   a down-regulation of STK11, LARP1, ZC3H12A, TFEB, TICAM1, GOLGA2, PIK3R2, IFI16,

221   ULK1, WDR81, SQSTM1 (also down-regulated at 48h), and HK2. As per viral infection, NIC

222   and virus treated cells (at 48h) also saw an up-regulation of GABARAPL1, MAP1LC3A,

223   USP30-ORG3a, BMF, and TBK1; WDR81 was also down-regulated in the NIC and virus

224   treatment at 48h.

225   There were significant transcriptomic differences noted at 16h and 48h with NIC treat-

226   ment (**Figures 6E and F**), and at 48h with NIC treatment and SARS-CoV-2 infection (**Table**

227   **S1B, Table S7, Figure 6G**. NIC treatment alone induced 2,447 DE genes at 16h (1,301 up-

228   regulated; 1,146 down-regulated), and 2846 genes at 48h (1,707 up-regulated; 1,139 down-

229   regulated), while viral infection and NIC treatment resulted in 3,617 DE genes at 16h (1,698

230   up-regulated; 1,919 down-regulated), although no DE genes were noted by 48h (**Figures 6E-**

231   **G**, **Table S7**). Overrepresented GO terms with NIC treatment related to autophagosome as-

232   sembly, regulation of autophagy, intracellular lipid metabolism, lipid localization and lipid ho-

233   meostasis (**Figure S6**). Similar genes were noted for the effect of NIC as were with the effect

234   of virus. RB1CC1 was up-regulated at 16h with virus infection (**Figure 6A**) but was down-

235   regulated upon NIC treatment (**Figure 6E**) at 16h. Conversely, BMF was down-regulated with

236   NIC at 48h, but was up-regulated upon NIC and virus treatment at 48 hours. Other genes that

237   were down-regulated with viral infection (GOLGA2, IFI16, WDR81, HK2, SQSTM1, ULK1) re-

238   mained down-regulated with NIC treatment (with and without virus).

239

240   **DISCUSSION**

241   A virus subjugates host lipid molecules as a vehicle for entry, intracellular transport, vi-

242   rus replication, assembly of infectious viral particles, and egress (Miyanari et al., 2007). Ether

243   lipids have been identified as an important lipid class for efficient membrane trafficking, endo-

244   cytosis, transcytosis, and internalization of particles (Thai et al., 2001; Deng and Angelova,

245   2021). Deficiency of ether lipids in several model systems affects plasma membrane function,

246   as well as structural changes in the ER and Golgi cisternae (Bazill and Dexter, 1990; Gorgas

247   et al., 2006). SARS-CoV-2 infection has demonstrated a diverse range of COVID-19 disease

248   severities that correlate with increased viral replication (Fajnzylber et al., 2020). Unlike during

249   the early infection stage at 16h, where virus load and replication is low, we observed that

250   SARS-CoV-2 infection uniquely elevated ether linked lipids after 48h of infection, a phase that

251   represents increased virus replication, virus assembly and egress as well as host cell energy

252   utilization (Abu-Farha et al., 2020; Dimitrov, 2004; Mazzon and Mercer, 2014). Increased viral

253   replication drives infection severity and may lead to a perturbation of the host cellular molecu-

254   lar landscape (Fajnzylber et al., 2020).

255   We observed that NIC treatment significantly downregulates the ether lipid profile during

256   infection. We also observed that NIC treatment in Vero E6 cells causes a reduction in TG con-

257   tent at both 16h and 48h suggesting a disruption of lipid droplet formation. (**Figure 4A**). NIC

258   has previously been tested as an anti-obesity drug, and a study using it associated with a high

259   fat diet showed a decrease in total TGs (Al-Gareeb et al., 2017). NIC also affects several lipid

260 species, including PE, which may stimulate the autophagy process and inhibit ether lipid eleva-

261 tion during viral propagation. However, NIC has dual activity in cells and is capable of influenc-

262 ing autophagic processes through canonical and non-canonical pathways (Liu, et al., 2019).

263 Whereas in uninfected cells, NIC reduces autophagic flux as well as the global lipid profile

264 (causing a decrease in plasmalogens, TGs and PE lipids), in infected cells, NIC induces au-

265 tophagy and the subsequent reduction of the ether lipid profile. NIC has been shown to affect

266 the function of lysosomes by preventing the acidification of the vacuole (Jung et al., 2019).

267 This disruption of lysosomal function results in increased autophagic flux similar to the likely

268 primary mechanism of action underlying the broad antiviral activity of chloroquine (Mauthe et

269 al., 2018). Importantly, we identified that ether linked lipids were only affected by NIC treat-

270 ment relative to productive virus infection at 48h, suggesting that the activity of NIC may be

271 dependent on the cellular state or level of virus burden and virus replication kinetics.

272 To understand the critical lipids related to autophagy activation, we analyzed the

273 phosphatidylethanolamine (PE) levels in NIC treated SARS-CoV-2 infected cells. PE is one of

274 the central lipids that conjugates the factors for induction of autophagy through the formation of

275 the autophagosome (Xie et al., 2020). We observed that the relative abundance of PE was

276 significantly elevated with NIC treatment at 48h while there was no impact of NIC on PE levels

277 at 16h, suggesting that NIC enhanced the autophagy machinery upon viral replication to direct-

278 ly affect downstream virus assembly, trafficking, and egress. Different signaling lipids such as

279 PS, PI, and PGs play critical roles in endosome trafficking and maturation (Mazzon and Mer-

280 cer, 2014) and help in infectious viral particle transport. Our study first identified that NIC

281 treatment significantly reduced the global level of different glycerophospholipids such as PS,

282 PI, and PG at 48h, but had no impact on early infection at 16h.

283   TGs are the major form of lipids enriched in lipid droplets. Drugs that stimulate TG

284   downregulation usually activate autophagy and may prevent a cell from viral infection and

285   pathogenesis. We identified that NIC treatment significantly reduced global levels of TGs in

286   SARS-CoV-2 at 48h treatment, suggesting that NIC treatment potentiates TG breakdown in

287   lipid droplets to help reduce viral replication. We also found that NIC treatment showed either

288   no effect or very low impact on lipids that were involved in lipid droplet formation at 16h. In

289   fasting conditions, lipid droplets are utilized for fatty acid oxidation to produce energy as intact

290   TGs are broken down to release fatty acids in a process called lipophagy. The breakdown of

291   TGs via hydrolysis is mediated by autophagy and we observed a decrease in TGs with NIC

292   treatment. Overall, these findings demonstrate that NIC treatment likely activates autophagy

293   and consequently impacts ether linked lipids and other lipids needed for viral infection and

294   propagation.

295   Bis(monoacylglycerol)phosphates (BMP), identified as a new lipid of endosome-derived

296   extracellular vehicles (EVs), also act as cholesterol transporters in cooperation with other fac-

297   tors and facilitate viral infection and replication (Luquain-Costaz et al., 2020). Interestingly, our

298   study detected three different forms of BMP and two different forms of cholesterol esters (CE)

299   that were all significantly downregulated with NIC treatment. Besides the impact of NIC on dif-

300   ferent autophagy related lipids, we also investigated the role of NIC on bioactive lipid metabo-

301   lism and apoptosis. We observed a dichotomous pattern of CerNS and SM lipids, where

302   CerNS decreased after NIC treatment (48hV vs 48hV+NIC) while SM were increased, indica-

303   tive of cell death or cell survival, which may be partly explained by the cell state-dependency of

304   NIC that we have observed in the presence or absence of SARS-CoV-2 infection in Vero E6

305   cells. Interestingly, we identified a notable reduction of gangliosides detected as HexCer

306 (which are also called glycosphingolipids) upon NIC treatment at 48h in both uninfected and

307 SARS-CoV-2 infected Vero E6. Gangliosides have been implicated in the induction of

308 autophagic death in mammalian cells (Hwang et al., 2010), suggesting that NIC can increase

309 cell survival at homeostasis as well as under stress/infection conditions. This activity may in

310 part contribute to the low cytotoxicity for this drug for several different cell lines, with the re-

311 ported CC_{50}= 50 µM (Jeon et al., 2020; Xu et al., 2020). Although gangliosides have also been

312 shown to bind cooperatively with ACE-2 to the SARS-CoV-2 spike protein (Fantini et al., 2021),

313 our experimental design did not include a pretreatment of cells prior to virus infection. The

314 search for highly safe drugs for prophylaxis against COVID-19 remains elusive and controver-

315 sial, but these data may suggest a role for niclosamide for future outbreaks given its broad an-

316 tiviral activities. Considering the potential link between plasmalogens and the observed cyto-

317 kine and lipid storms (Deng and Angelova, 2021) in severe COVID-19 patients, NIC may offer

318 to a two-pronged treatment approach, i.e., reduce virus abundance and provide a check on an

319 uncontrolled inflammatory response induced by SARS-CoV-2.

320 These findings are further supported by the global transcriptome profile that we cap-

321 tured for each of the treatments/conditions, wherein hallmarks of and the induction of autopha-

322 gy related genes (such as DEPP1 (an autophagy regulator) (Salcher et al., 2017)), several li-

323 pases (Gassen et al., 2021), as well as genes implicated in the induction of apoptosis and

324 genes that are markers of cell proliferation. Furthermore, GO overrepresentation analyses

325 showed significantly upregulated terms such as death receptor activity, MAP kinase phospha-

326 tase activity, tumor necrosis factor-activated receptor activity, and phosphatidylinositol kinase

327 activity. To investigate the role NIC has on the reversal of SARS-CoV-2 infection induced au-

328 tophagy dysregulation, we explored the function of several genes that were differentially ex-

329   pressed. RB1CC1 (RB1 inducible Coiled-Coil 1) along with ULK1 are part of the ULK complex

330   that initiates and regulates autophagy (Zachari and Ganley, 2017). RB1CC1 has also been

331   shown to influence viral infection (Mauthe and Reggiori, 2016), as a depletion of RB1CC1 led

332   to an increase of encephalomyocarditis virus replication. Here, RB1CC1 was up-regulated by

333   SARS-CoV-2 at 16h; however, was significantly down-regulated with treatment of NIC at 16h.

334   We also observed down-regulation of ULK1 in NIC treated cells at 16h. This elucidates the po-

335   tential role of RB1CC1 and ULK1 in early infection with SARS-CoV-2 and illuminates how NIC

336   may regulate autophagy. Interestingly, BMF was down-regulated at 48h with NIC, but up-

337   regulated upon NIC treatment of virus infected cells at 48h. BMF encodes for a Bcl-2-modifying

338   factor that is responsible for apoptotic regulation and has been reported as a target facilitating

339   viral evasion (Zamaraev et al., 2020). Additionally, autophagy genes IFI16 (Duan et al., 2011;

340   Jiang et al., 2021; Kim et al., 2020; Wichit et al., 2019), ZC3H12A (Srivastava et al., 2020),

341   SQSTM1 (Gassen et al., 2021), WDR81 (Zhu et al., 2021), and PIK3R2 (Weisberg et al.,

342   2020) are associated with viral infection, including SARS-CoV-1 (Gassen et al., 2021;

343   Srivastava et al., 2020) and were downregulated in this study when cells were treated with

344   NIC. Taken together, this provides evidence that SARS-CoV-2 infection leads to dysregulation

345   of autophagy, and that NIC acts to reverse this effect.

346   Several omics studies have made a significant contribution to the identification and un-

347   derstanding of potential antiviral therapies for COVID-19 (Mahmud and Garrett, 2020). Bioac-

348   tive molecules including PUFA lipids such as oleoylethanolamide (OEA), arachidonic acid

349   (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) are known from existing

350   viral pathogenesis to inactivate enveloped viruses and inhibit pathogen proliferation/replication

351   (Das, 2020; Ghaffari et al., 2020; Tam et al., 2013). Accumulation of lipids including

352 sphingolipids such as ceramides has been shown to negatively affect viral pathogenesis

353 (Soudani et al., 2019; Young et al., 2013). Lipid metabolism; specifically catabolism, biosyn-

354 thesis, and peroxidation play critical roles in autophagy or apoptosis-mediated cellular homeo-

355 stasis including cell survival and death (Xie et al., 2020). Autophagy can act as an anti-viral or

356 pro-viral mechanism; however, most viruses are found to inhibit autophagy signaling (Jackson,

357 2015). In the case of SARS-CoV-2, we know little about the relationship of virus infection with

358 autophagy signaling. Importantly, we identified elevated levels of ether lipids (plasmanyl and

359 plasmenyl) with increased viral replication, which was reported as a key lipid class for efficient

360 membrane trafficking (Thai et al., 2001). We revealed that lipids are critical molecular factors

361 for SAR-CoV-2 infection, entry, and viral replication into host cells. These lipids, which were

362 elevated by SARS-CoV-2 infection may be counteracted by PUFA and VLCFA that the virus

363 suppresses upon infection and entry (Kang et al., 2019).

364   Collectively, these data provide new insight into the mechanism of action for the antiviral

365 activity of NIC, namely induction of lipophagy through the induction of autophagic pathways

366 (**Figure 7**), which expands as well as refines our understanding of the pleiotropic antiviral

367 mechanisms described for NIC (Braga, et al., 2021; Gassen, et al., 2021; Kao, et al., 2018).

368 We can mechanistically infer that SARS-CoV-2 infection wrests control of cellular autophagy to

369 ensure a productive infection of the host cell. Therapeutically, we demonstrated that NIC

370 treatment stimulated autophagy by elevating PE levels and in parallel, downregulated total

371 ether lipids, TGs, and other lipid molecules. Reduction of these key lipids by NIC treatment

372 may lead to inhibition of SARS-CoV-2-induced signaling, which in turn drives viral endocytosis,

373 vesicular trafficking, propagation, and viral egress. Activating autophagy using small molecule

374 inhibitors/activators, oxysterols, or peptides is a promising approach for treating viral infections

375   (Rubinsztein et al., 2012). However, most of the potential treatments (including NIC) suffer

376   translational obstacles due to adverse side effects, inefficient drug delivery to target tissues,

377   and poor bioavailability (Skotland et al., 2015). Although NIC is considered an essential medi-

378   cine and is well tolerated, the long history of dosing safety is based on *per os* delivery and anti-

379   parasitic activity within the gastrointestinal tract. SARS-CoV-2 infection begins in the lungs but

380   has been shown in several clinical studies to have a cosmopolitan infectivity profile, including

381   the brain, heart, and gastrointestinal tract. Notably, several repurposed drugs for COVID-19

382   were found to be associated with autophagy induction (Kim et al., 2012), raising the possibility

383   that some drugs that are already in clinical use for the treatment of parasitic or bacterial infec-

384   tions may be acting, at least in part, via autophagy. The recent clinical testing of intranasal or

385   inhaled NIC (Backer et al., 2021) suggests that the potential of this drug for use in mitigating

386   COVID-19 severity is profound, compeling expansive exploration of different approaches to

387   increase targeted delivery and efficacy.

388

389   **Limitations**

390   Although we used a *C. sabaeus* kidney epithelial cell line (Vero E6) and a SARS-CoV-2

391   strain isolated from a Floridian patient, we observed a similar (in direction and magnitude)

392   transcriptomic response to other SARS-CoV-2 transcriptomic studies, despite differences in

393   MOI and experimental sampling timepoints. These changes were consistent across another

394   Vero E6 study noting cell stress and apoptosis (DeDiego et al., 2011), as well as other cell sys-

395   tems, including human lung cells (Wyler et al., 2021), cardiomyocytes (Sharma et al., 2020),

396   adenocarcinomic human alveolar basal epithelial cells (Daamen et al., 2021), and bronchial

397   epithelial cells (Yoshikawa et al., 2010), infected with different betacoronaviruses, suggesting

398   the responses detected herein may represent a core set of host SARS-CoV viral response

399   genes, and that these genes are not exclusive to our study system. Our work is limited to an *in*

400   *vitro* model; however, it was recently shown in a multiomics study of COVID-19 patient sam-

401   ples that the lipidomic profile can effectively partition COVID-19 disease severity (Overmyer et

402   al., 2021); implying that the biology captured in our culture model is relevant *in vivo*. While

403   these observations highlight potential utility of niclosamide, these data alone do not support its

404   indication in the clinic in the treatment of COVID-19.

405

406   **STAR METHODS**

407   **RESOURCE AVAILABILITY**

408   **Lead Contact**

409   Further information and requests for resources and reagents should be directed to and will be

410   fulfilled by the lead contact, Dr. Timothy Garrett (tgarrett@ufl.edu).

411   **Materials availability**

412   This study did not generate new unique reagents.

413   **Data and code availability**

414   The raw and processed data generated here have been deposited in publicly accessible data-

415   bases; the RNA sequencing data is available through NCBI's GEO repository (Accession:

416   GSE178157), and the lipidomics data is accessible via MetaboLights (Accession:

417   MTBLS2943).

418   This paper does not report original code.

419   Any additional information required to reanalyze the data reported in this paper is available

420   from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Vero E6 cells.

Vero clone E6 cells (ATCC: CRL-1586) were obtained from Dr. Pei-Yong Shi (University of

Texas Medical Branch). Vero E6 cells are an immortalized kidney epithelial cell line obtained

from the African green monkey (*Cercopithecus sabeus*). The cells are female.

Cells were maintained at 37 °C and 5% CO_2, in Dulbecco's Modified Eagle Medium

(ThermoFisher #11965118) supplemented with 10% heat-inactivated fetal bovine serum

(FBS), 1X L-glutamine (Gibco #25030-081), and 1X Penicillin/Streptomycin (Corning #30-001-

CI). These cells have been authenticated.

SARS-CoV-2.

SARS-CoV-2/human/USA/UF-13/2020 (GenBank: MT620766.1) was passaged in Vero E6

cells, with culture conditions as described above except using reduced serum media with 3%

FBS rather than 10% FBS. Virus was collected after 4 days to establish a low passage virus

stock within our biosafety level 3 (BSL-3) laboratory at the Emerging Pathogens Institute.

METHOD DETAILS

Cell culture, infection, and niclosamide treatment.

Cells were seeded in 6 well plates at a density of 500,000 cells/well. Each replicate sample

represents an individual plate well, with three biological replicates per condition, per time point.

The investigators were not blinded to the conditions. At hour zero (48 hours post seeding),

media was removed and replaced with reduced serum Dulbecco's Modified Eagle Medium

441   (supplemented with 3% FBS, 1X L-glutamine, and 1X Penicillin/Streptomycin) to facilitate in-

442   fection.

443   One vial of SARS-CoV-2 stock was thawed and diluted to a titer of 2×10^7 plaque form-

444   ing units (PFU) per mL. For infected conditions, virus was inoculated into each well with 12.5

445   μL of diluted virus stock for a multiplicity of infection (MOI) of 0.5 infectious virus particles/ Vero

446   E6 cell. Niclosamide (MedChemExpress #HY-B0497, [NIC]) stock was dissolved in molecular

447   grade DMSO and added to the indicated wells for a final concentration of 5 μM (0.5% v/v

448   DMSO final concentration). An equivalent amount of molecular grade DMSO was used as a

449   vehicle control. This concentration of NIC was selected to have antiviral effect but be well be-

450   low the observed 50% cytotoxic concentration (CC_{50}) of the drug (50μM) to avoid inadvertent

451   cell lysis in treated conditions (Wu et al., 2004).

452   Samples were prepared and collected following a time of addition experimental design

453   (Daelemans et al., 2011) as follows (**Figure 1, Table S1A-B**). The "0 hour" (0h) samples were

454   collected 48h after cell seeding. The "16 hour" (16h) conditions were infected with virus, treat-

455   ed with NIC or DMSO at hour zero, and harvested at hour 16 of the experiment (**Figure 1**).

456   The "48 hour" (48h) conditions were infected with virus at hour zero, supernatant was removed

457   after 24h, and cells were treated with NIC or DMSO in fresh media. Cells were harvested at

458   hour 48. Uninfected samples (with and without drug treatment) were also prepared and col-

459   lected as above. For samples from infected conditions, the supernatant was removed at the

460   indicated harvest timepoint, taking care not to disturb the cell monolayer, and retained at -

461   80°C. The cells were washed once with 1X PBS. The PBS wash was removed and 0.25%

462   trypsin-EDTA (ThermoFisher #25200056) was added to each well. The plates were then incu-

463   bated at 37°C and 5% CO_2 for 5-10 minutes until cells had completely detached from the cell

464 surface. Trypsinization was halted by the addition of 1 mL FBS. The supernatant was re-

465 moved, the cell pellet was resuspended in 1X PBS, and the sample was centrifuged at 700

466 RPM for 5 minutes. Supernatant was aspirated, and 100% methanol was added to each sam-

467 ple purposed for lipid analysis. Alternatively, 750 µL of TRIzol reagent (ThermoFisher

468 #15596026) was added to each sample purposed for RNASeq. All samples were stored at -

469 80°C until analysis. For uninfected conditions, harvest proceeded the same as described

470 above except trypsinization was halted using 4 mL of complete Dulbecco's Modified Eagle

471 Medium instead of FBS, and pellets were flash frozen in liquid nitrogen instead of being resus-

472 pended in a chemical inactivation agent after the last centrifugation step. All samples were

473 stored at -80°C until analysis.

474 **Global lipid extraction.**

475 Cell pellets from each time point and condition were extracted using a modified Folch biphasic

476 extraction procedure (Ulmer, et al. 2017). Samples were pre-normalized to the protein concen-

477 tration (800 µg/mL) obtained using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific). Lipids

478 were extracted using ice cold 4:2:1 chloroform:methanol:water (v:v:v) containing 20 µL of a

479 10X diluted internal standard mixture (stock solution of 50 ppm, w: v), and the organic phase

480 was collected after centrifugation at 3260 xg for 5 min at 4°C, dried under nitrogen gas, and

481 reconstituted in 75 µL of Isopropanol (IPA) plus 1 µL of injection standard mixture (100 ppm,

482 w:v). Internal standards used in this analysis covered a range of lipid classes and structures

483 including: lysophosphatidylcholine (LPC 17:0), phosphatidylcholine (PC 17:0/17:0),

484 phosphatidylethanolamine (PE 15:0/15:0), phosphatidylserine (PS 14:0/14:0),

485 phosphatidylglycerol (PG 14:0/14:0), ceramide (Cer d18:1/17:0), diacylglycerol (DG 14:0/14:0),

486 and sphingomyelin (SM d18:1/7:0). For injection standards, triacylglycerol (TG 17:0/17:0/17:0),

487   LPC 19:0, PE 17:0/17:0, PS 17:0/17:0, and PG 17:0/17:0 were used. Except for TG, all other

488   lipid standards were purchased from Avanti Polar Lipids (Alabaster, AL) while TG was pur-

489   chased from Sigma Aldrich. All lipid standards were diluted prior to analysis in 1:2 (v/v) chloro-

490   form:methanol (CHCl$_3$:MeOH) and a working standard mix was then prepared by diluting the

491   stock solution with the same solvent mixture.

492   **LC-MS-based lipid data collection and analysis.**

493   Ultra-high-pressure liquid chromatography coupled to high resolution mass spectrometry

494   (UHPLC-HRMS) was used for data collection. Chromatographic separation was achieved us-

495   ing reversed phase chromatography (Dionex Ultimate 3000 RS UHLPC system, Thermo Sci-

496   entific) with a Waters Acquity C18 BEH column (2.1 × 50 mm, 1.7 µm) (Waters, Milford, MA,

497   US). The mobile phases consisted of solvent A (60:40 acetonitrile:water) and solvent B

498   (90:8:2) isopropanol:acetonitrile:water), both containing 10 mM ammonium formate and 0.1%

499   formic acid. The flow rate was 500 µL/min and the column temperature was maintained at

500   50°C. A multi-step gradient was used for separation starting with 0% B from 0-1 min, increas-

501   ing to 30% B from 1-3 min, then up to 45% B from 3-4 min, 60% B from 4-6 min, 65% B from

502   6-8 min, held at 65% B from 8-10 min, increased to 90% B from 10-15 min, then increased to

503   98% B from 15-17 min and finally held at 98% B from 17-18 min before returning to initial con-

504   ditions to equilibrate for the next injection. Samples were analyzed in positive and negative

505   electrospray ionization as separate injections on a ThermoScientific Q-Exactive high resolution

506   mass spectrometer (Thermo Scientific, San Jose, CA). Lipidomics data were compiled and an-

507   notated using LipidMatch (Koelmel et al., 2017).

RNA extraction.

For total RNA extraction from flash frozen cells, the cell pellet was thawed on ice. Cell pellets were resuspended in 1 mL Trizol, and 200 µL chloroform was added to each sample. Samples were mixed thoroughly by vortexing, then incubated at room temperature for 3 minutes. Samples were centrifuged at 12,000 xg for 15 minutes at 4°C. The upper aqueous layer was transferred to a new tube and 500 µL isopropanol was added and mixed by vortexing, prior to incubation at room temperature for 10 minutes. RNA was pelleted by centrifuging at 12,000 xg for 10 minutes at 4°C, and supernatant was discarded. The pellet was rinsed in 75% ethanol and pelleted by centrifuging at 7,500 xg for 5 minutes at 4°C. The ethanol wash and spin were repeated a second time. The pellet was air dried in an inverted tube. Genomic DNA was digested using the TURBO DNA-free™ Kit (Invitrogen #AM1907) according to manufacturer instructions. Samples were mixed with 350 µL buffer RLT from the RNeasy® Mini kit (Qiagen #74104) and 250 µL 100% ethanol, then transferred to a RNeasy® Mini kit spin column. Columns were centrifuged at 8,000 xg for 15 seconds at room temperature, and flow-through was discarded. Buffer RPE (500 µL) was added to the column, which was centrifuged at the same conditions, and flow-through was discarded. The RPE wash was repeated a second time. The column was transferred to a fresh collection tube and centrifuged at 12,000 xg for 1 min to dry. RNA was eluted by adding 50 µL of nuclease free water and centrifuging for 1 minute at 8,000 xg and was immediately frozen at -80°C. To extract viral RNA from culture supernatant, supernatant was thawed, 200 µL of supernatant was mixed with 200 µL DNA/RNA Shield, and RNA was immediately extracted using the Quick-DNA/RNA™ Viral MagBead kit (Zymo Research #R2140), according to manufacturer instructions and then immediately frozen at -80°C.

Real-time RT-qPCR.

To quantify viral genome copy in supernatant, one step real time reverse transcription qPCR (RT-qPCR) was performed using 4x Quantabio UltraPlex 1-Step ToughMix No Rox (VWR #10804-946) and CDC 2019-nCoV_N1 (nucleocapsid) primer and probe mix (see Key Resources Table for primer and probe sequences) at a final concentration of 22.5 µM, in a 20 µL reaction volume with 5 µL template. All samples were run in technical duplicates. RT-qPCR was run on a BioRad CFX96™ Real-Time System. The thermal cycling conditions were as follows: 50°C for 20 minutes, 94°C for 2 min; followed by 45 cycles of 94°C for 15 seconds, 55°C for 30 seconds, and 68°C for 10 seconds. The samples were quantified using a standard curve generated using a 2019-nCoV_N_Positive Control plasmid (IDT # 10006625). Standard curve points were plotted in Microsoft Excel v.2102 and cycle threshold values of unknown samples were determined from the logarithmic line of best fit equation to calculate genome copies/mL.

RNASeq analysis.

All 27 RNA samples (see **Table S1A-B**) were directly sent to Novogene (https://en.novogene.com/), where in-house RNA quality control and library preparation was performed. Libraries were sequenced on an Illumina NovaSeq6000 platform using a 150bp kit with paired-end read mode.

Mapping, expression, and pathway analyses.

Bioinformatic processing was completed using Galaxy (https://galaxyproject.org, (Batut et al., 2018)). Quality control was performed with Cutadapt v 1.16 (Martin, 2011) and FastQC v 0.11.8 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to remove adapters, <20 nucleotide reads and low-quality reads. RNA STAR v2.7.7a (Dobin et al., 2013) was used to map samples to the *Chlorocebus sabaeus* genome and associated annotation (GenBank ac-

553 cession # GCA_015252025.1). featureCounts v 2.0.1 (Liao et al., 2014) was used with Infer

554 Experiment v2.6.4.1 (Wang et al., 2012) to determine the strandedness of the samples, and

555 sum reads for each gene. Read counts for each sample were input into DESeq2 v 1.22.1

556 (Love et al., 2014) to call differential gene expression, analyzing the effect of time, drug, and

557 virus on the samples. Resultant differential expression files were manually annotated using a

558 combination of NCBI (O'Leary et al., 2016) and Ensembl (Yates et al., 2020) due to the poor

559 annotation   quality   of   the   genome

560 (https://www.ncbi.nlm.nih.gov/genome/annotation_euk/Chlorocebus_sabaeus/100/).   Signifi-

561 cantly regulated gene names were separately parsed out for each comparison and uploaded to

562 ENRICHR (Kuleshov et al., 2016) for downstream gene ontology (GO (Harris et al., 2004)) and

563 pathway analyses using Reactome (Fabregat et al., 2016), MSigDB (Liberzon et al., 2015),

564 and KEGG (Ogata et al., 1999)) databases.

QUANTIFICATION AND STATISTICAL ANALYSIS

566 Unless indicated in the figure legend, all experiments were performed in triplicate and results

567 are presented as mean ± standard error of mean (SEM) of absolute values or percentages of

568 control. Each replicate was a separate tissue culture well processed in parallel. A list of all

569 samples used for subsequent analysis can be seen in **Table S1A-B**.

570 Statistical P-values were obtained by application of the appropriate statistical tests using

571 GraphPad Prism v.9.0. Lipidomics data was normalized to the total ion signal, glog trans-

572 formed, autoscaled and analyzed with Metaboanalyst v.3.0 (Pang et al., 2021). For quality con-

573 trol, internal lipid standards added to each sample were used to assess technical reproducibil-

574 ity, achieving ≤15% relative standard deviation (RSD) across all samples. Correlation

575   heatmaps were generated for the lipid samples using Pearson's r. Heatmap clustering was

576   performed using a T-test or an ANOVA, showing the most significant lipids. For lipidomics ap-

577   plications, Bonferroni false discovery rate (FDR) adjusted P-values lower than 0.05 and an ab-

578   solute fold-change greater than or equal to 1.5 were considered significant. For RNASeq dif-

579   ferential gene expression, gene ontology and pathway analyses, statistical significance was

580   corrected for multiple comparisons (Bonferroni adjusted) and assessed at an $\alpha=0.05$. Genes

581   with absolute $\log_2$ fold changes ≥ 1 were considered significantly differentially expressed. For

582   supernatant genome copy RT-qPCR, a two-way non-parametric ANOVA with Dunn's post-hoc

583   test was performed in GraphPad Prism v.6.0 and assessed at an $\alpha=0.05$. Since no statistical

584   differences were observed between technical replicates of the same condition in the superna-

585   tant genome copy RT-qPCR data, technical replicate wells were pooled in the reported analy-

586   sis.

587   **AUTHOR INFORMATION**

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591

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595

596   **Author Contributions**

597 The manuscript was written through contributions by all authors. RRD, TG and JAL conceptu-

598 alized the study. IM, HC, TH, CJS, and JA conducted the study and generated samples and

599 data. IM, HC, TH, MM, JA, HSY, RRD and TG analyzed the data. IM, HC, TH, MM, JA, JAL,

600 RRD and TG wrote/edited the manuscript. All authors have given approval to the final version

601 of the manuscript.

602 **‡These authors contributed equally.**

603 **Declaration of Interests**

604 The authors declare no competing interests.

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871

FIGURE LEGENDS

873 **Figure 1. Workflow for lipidomic and transcriptomic profiling of Vero E6 cells after**

874 **SARS-CoV-2 infection with and without niclosamide treatment.**   We used a time-of-

875 addition assay experimental design to (i) capture the lipidomic profile of SARS-CoV-2 infected

876 Vero E6 cells, and (ii) explore the effect of niclosamide on the lipidomic profile of Vero E6 cells

877 when added in the absence of infection, with SARS-CoV-2 virus, or at 24h post-infection. Each

878 replicate experimental condition (n=3) was processed for LC-HRMS/MS or RNASeq analyses.

879 Samples were seeded 48h prior to the start of the experiment (t = 0h). For all samples, media

880   was changed at the start of the experiment (t = 0h) and infected with virus (for infected sample

881   groups). Sample collection is denoted by an up arrow and tube above the timeline, media

882   changes are denoted by red bottles, addition of virus and DMSO/drug are denoted as well.

883   Separate samples for each condition were collected for LC-HRMS/MS and RNAseq analysis,

884   respectively.

885

886   **Figure 2. Global lipidomics analysis in SARS-CoV-2 infected Vero E6 cells at 16h and**

887   **48h.** (A) Hierarchical cluster heatmap analysis depicting the major affected lipid clustering be-

888   tween 16h and 48h infection. (B) Volcano plot showing the differential lipid abundance with

889   SARS-CoV-2 infection between 16h and 48h. The primary significant upregulated lipids were

890   ether-linked (plasmanyl and plasmenyl). (C) Bar graph showing the total abundance of ether

891   lipids between the time points. (D) Extracted ion chromatogram comparison showing relative

892   peak intensity of a single ether lipid between 16h (blue) and 48h (magenta) infection. The ar-

893   row points to the lipid identified as Plasmanyl-PC (O-18:0/16:0)+H at retention time 9.16. Iden-

894   tification was confirmed by accurate mass and MS/MS.

895

896   **Figure 3. Comparison of individual lipid classes at early (16h) and late (48h) viral infec-**

897   **tion. FDR P-value based on T-test shown above each plot.** Individual triangles represent

898   each lipid detected and expression compared at both time points. Abbreviations: TG-

899   triglycerides, DG-diglycerides, CL-cardiolipin, CE-cholesterol ester, SM-sphingomyelin, PC-

900   phosphatidylcholine, PE-phosphatidylethanolamine, PS-phosphatidylserine, PI-

901   phosphatidylinositol, PG-phosphatidylglycerol, LPC-lysophosphatidylcholine, LPE-

902   lysophosphatidylethanolamine, oxLPC-oxidized lysophosphatidylcholine, HexCer-

903   hexosylceramide, CerNS-ceramide with non-hydroxy fatty acid, n.s.-not significant.

904

905   **Figure 4. Niclosamide modulates lipid metabolism in Vero E6 cells in the absence of**

906   **SARS-CoV-2 infection.** Heatmap showing the changes in neutral lipids across the time points

907   with and without NIC (A). Bar graphs of the significant lipids at 16h vs 48h (B) and 48h vs 48h

908   + NIC (C). Fold change of less than 1.5 and p-value less than or equal to 0.05.

909

910   **Figure 5**. **Niclosamide (NIC) impact ether-linked lipid production during viral replication**

911   **at 48h.** (A) Heatmap of the top 70 lipids (Fold change $\geq$ 1.5 and p-value $\leq$ 0.05). (B) Volcano

912   plot showing the significant up and down regulated lipids with NIC at 48h. (C) The total ether

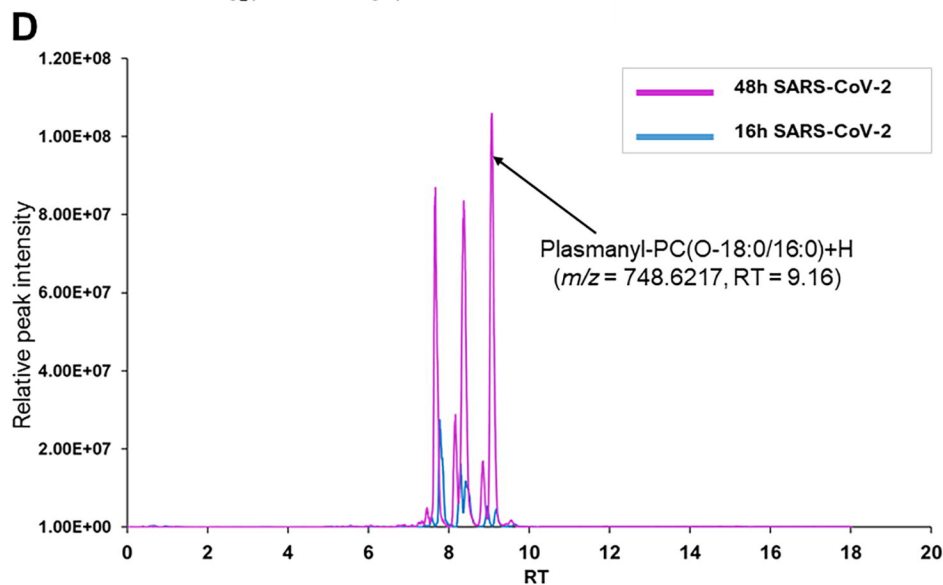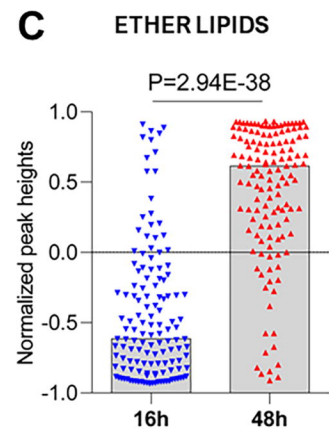913   lipids were down regulated with NIC treatment.

914

915   **Figure 6. Significantly expressed genes across SARS-CoV-2 and Niclosamide condi-**

916   **tions.** Genes were designated as significantly expressed using DESeq2 when their Bonferroni

917   adjusted P-value was ≤0.05, and the |log2(Fold Change)| was ≥1. Each dot represents an indi-

918   vidual gene. Blue dots are significantly downregulated genes, red dots are significantly

919   upregulated genes, and grey dots are not significantly differentially expressed genes. (A)

920   DMSO treated no virus versus DMSO treated, virus samples at 16 hours, (B) DMSO treated no

921   virus versus DMSO treated virus samples at 48 hours, (C) Niclosamide treated no virus versus

922   niclosamide treated virus samples at 16 hours, (D) Niclosamide treated no virus versus

923   niclosamide treated virus samples at 48 hours, (E) DMSO treated no virus versus niclosamide

924   treated no virus samples at 16 hours, (F) DMSO treated no virus versus niclosamide treated

925   no virus samples at 48 hours, (G) DMSO treated virus versus niclosamide treated virus sam-

926   ples at 16 hours, (H) DMSO treated virus at 16 hours versus 48 hours, (I) Niclosamide treated,

927   no virus samples at 16 vs 48 hours.

928

929   **Figure 7. The effect of SARS-CoV-2 infection and NIC treatment on host cell lipid me-**

930   **tabolism.** SARS-CoV-2 infection in Vero E6 cells alters host cell lipid metabolism during early

931   and late stages of infection. Increased transcription of lipid receptors LRP2 and VLDLR, and

932   phosphorylation signaling regulators are observed throughout viral infection. Changes in TG

933   composition from unsaturated to saturated acyl-chains occurs as a function of viral replication,

934   with an overall decrease in TG lipids at late infection timepoints. This change corresponds with

935   an increase to DG and BMP lipids that is indicative of energy consumption and incorporation

936   into membranes and vesicles, activation of autophagy pathways, as well as impacting viral rep-

937   lication. Treatment of cells with NIC alters lipid composition and gene regulation corresponding

938   to apoptosis and autophagy related pathways. Decreases to ether lipids (TGs and DGs) and

939   BMP are observed and reflect a decrease to exocytosis pathways for viral egress.

940

Time-of-Addition Assay

Co-inoculation with treatment

Treatment post-inoculation

-48h   0h   16h

-48h   0h   24h   48h

16 h   16 h

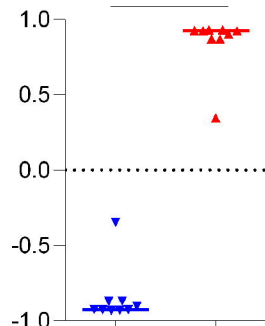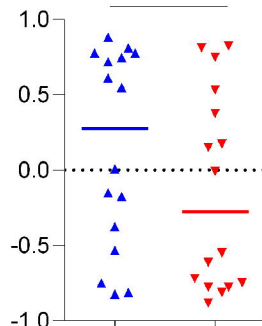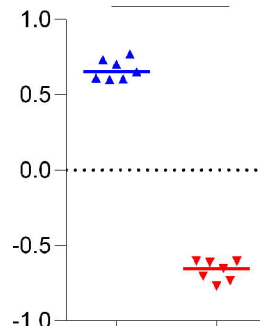16 h   16 h

48 h   48 h

48 h   48 h

Data Acquisition

LC-HRMS/MS   RNASeq

Expression & Pathway Analyses

A — Heatmap: 16h SARS-CoV-2 | 48h SARS-CoV-2. Log2 FC scale from -2.5 to 2.5.

TG(18:1_22:1_22:4)
TG(20:4_22:1_22:1)
TG(20:2_22:3_22:4)
TG(18:1_22:2_22:4)
TG(18:1_22:4_22:4)
TG(18:1_20:1_22:4)
TG(18:1_20:2_22:2)
TG(18:1_20:2_22:4)
CL(36:3)(36:3)
Plasmanyl-LPC(O-20:1)
LPC(16:0)
TG(22:3_22:3_22:3)
TG(17:0_20:2_20:3)
TG(16:0_20:2_25:0)
HexCer-NS(d18:1/22:0)
PG(16:0_16:0)
Cer-NS(d18:1/16:0)
HexCer-NS(d18:1/24:0)
Plasmenyl-PE(P-16:0/20:5)
plasmanyl-TG(O-16:1_20:0_20:0)
PE(44:1)
Cer-NS(d18:1/22:1)
DG(18:1_18:1)
DG(16:0_18:1)
plasmanyl-TG(O-16:0_18:1_20:1)
plasmanyl-TG(O-16:0_18:1_20:2)
DG(16:1_18:1)
plasmanyl-TG(O-16:0_18:1_22:2)
DG(18:0_20:4)
Plasmanyl-LPC(O-16:0)
Cer-NS(d18:1/24:0)
PG(16:1_18:1)
Plasmanyl-PC(O-18:1/20:4)
plasmanyl-TG(O-18:0_16:0_18:1)
Plasmanyl-PC(O-16:0/14:1)
Plasmanyl-PC(O-16:0/16:1)
Plasmanyl-PC(O-16:0/17:0)
plasmanyl-TG(P-16:1_22:0_22:0)
Plasmanyl-PC(O-16:0/14:0)
DG(18:1_20:1)
Cer-NS(d18:1/24:1)
Plasmanyl-PC(O-16:0/18:1)
Plasmanyl-PC(O-16:0/22:1)
Plasmanyl-PC(O-16:0/22:2)
Plasmanyl-PC(P-20:1/18:1)
Plasmanyl-PC(O-16:0/14:0)
Plasmanyl-PC(O-18:0/16:0)
PC(18:1_24:1)
Plasmanyl-PC(O-18:1/16:1)
LPC(24:1)
DG(18:1_20:2)
plasmanyl-TG(O-18:1_16:0_18:1)
Cer-NS(d18:1/25:1)
SM(d18:2/16:0)
Plasmanyl-PC(P-16:1/18:0)
Plasmanyl-PC(O-18:0/18:1)
Plasmanyl-PC(O-18:1/22:2)
Plasmanyl-PC(P-16:1/18:1)
PC(16:1_18:2)
Plasmenyl-PC(P-16:0/22:1)

B

48h SARS-CoV-2 / 16h SARS-CoV-2

Labels: TG(20:2_22:3_22:4), TG(18:1_22:1_22:4), TG(18:1_22:2_22:4), TG(20:4_22:1_22:1), Plasmanyl-TG(O-16:0_18:1_20:1), TG(18:1_20:1_22:4), TG(18:1_22:4_22:4), Plasmanyl-PC(O-16:0/22:1), DG(18:1_20:1), DG(18:1_18:1), Plasmanyl-PC(O-18:0/16:0), Plasmanyl-PC(O-16:0/14:...)

x-axis: log$_2$(Fold Change); y-axis: -log$_{10}$(FDR p-value)

C — ETHER LIPIDS

P=2.94E-38

y-axis: Normalized peak heights; x-axis: 16h, 48h

D

48h SARS-CoV-2 (magenta)
16h SARS-CoV-2 (blue)

Plasmanyl-PC(O-18:0/16:0)+H
(*m/z* = 748.6217, RT = 9.16)

x-axis: RT; y-axis: Relative peak intensity

A

SARS-CoV-2 | SARS-CoV-2 + NIC

PC(16:1_20:4)
HexCer-NS(d20:1/15:0)
PC(16:1_20:4)
PC(13:0_20:3)
PE(42:10)
PC(14:0_14:1)
PE(20:4_20:4)-H
HexCer-NDS(d18:0/14:0)
PE(30:1)
PEtOH(40:9)
PC(16:1_22:6)
Plasmenyl-PE(P-16:0/20:5)
PC(18:2_20:4)
PC(18:1_20:5)
PC(16:1_22:6)
LPC(14:1)
SM(d18:1/14:0)
plasmenyl-TG(P-16:1_22:0_22:0)
Plasmanyl-PC(O-18:1/22:2)
PC(18:1_20:1)
Plasmanyl-PC(O-18:1/20:4)
DG(16:0_16:1)
Plasmanyl-PC(O-16:0/16:1)
Plasmanyl-PC(O-16:0/17:0)
Plasmanyl-PC(O-16:0/18:1)
Plasmanyl-PC(P-18:0/18:1)
Plasmanyl-PC(O-16:0/22:1)
PG(16:1_18:1)
plasmanyl-TG(O-18:1_16:0_18:1)
LPC(24:1)
Plasmanyl-PC(P-18:0/18:1)
HexCer-NS(d18:1/14:0)
HexCer-NS(d18:1/18:0)
plasmanyl-TG(O-16:0_16:0_18:1)
plasmanyl-TG(O-16:0_18:1_20:1)
Plasmanyl-PC(O-18:1/20:4)
plasmanyl-TG(O-18:0_16:0_18:1)
plasmanyl-TG(O-18:0_16:0_20:1)
Plasmanyl-PC(O-16:0/22:4)
plasmanyl-TG(O-16:0_18:1_20:2)
plasmanyl-TG(O-16:0_18:1_22:2)
plasmanyl-TG(O-16:0_18:1_22:6)
plasmanyl-TG(O-16:1_20:0_20:0)
Cer-NS(d18:1/22:0)
Cer-NS(d18:1/22:1)
Cer-NS(d18:1/23:0)
Cer-NS(d18:1/24:0)
Cer-NS(d18:1/24:0)
plasmanyl-TG(O-18:1_16:0_16:1)
plasmanyl-TG(O-18:1_16:0_22:1)
plasmanyl-TG(O-20:0_20:2_20:2)
plasmanyl-TG(O-18:0_18:1_22:1)
PG(17:1_17:1)
plasmanyl-TG(O-18:0_16:0_22:1)
DG(18:0_20:4)
DG(18:1_18:1)
plasmanyl-TG(O-18:0_18:1_22:4)
DG(16:1_18:1)
Plasmanyl-PC(O-18:0/16:0)
Cer-NS(d18:2/24:1)

B

SARS-CoV-2 + NIC / SARS-CoV-2

Plasmanyl-TG (O-18:1_16:0_18:1)
Plasmanyl-PC (O-18:1/20:4)
Plasmanyl-TG (O-16:0_18:1_20:2)
Plasmanyl-TG (O-16:0_18:1_20:1)
Plasmanyl-TG (O-18:1_16:0_22:1)
Plasmanyl-TG (O-16:0_16:0_18:1)
PC(14:0_14:1)
PE(20:4_20:4)-H
PC(16:1_22:6)
PC(13:0_20:3)CO_2
PC(16:1_20:4)

$-\log_{10}$(FDR p-value)

$\log_2$(Fold Change)

C

ETHER LIPIDS

P=2.49E-38

Normalized peak heights

SARS-CoV-2 | SARS-CoV-2+NIC