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More than a Mutagenic Aflatoxin B1 Precursor: The Multiple Cellular Targets of Versicolorin a Revealed by Global Gene Expression Analysis

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1 **More than a mutagenic Aflatoxin B1 precursor: the multiple cellular targets of**
2 **Versicolorin A revealed by global gene expression analysis**

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24 **Abstract**

25 Versicolorin A (VerA), a precursor of the potent carcinogen aflatoxin B1 (AFB1), is an
26 emerging mycotoxin. Recent research has highlighted the mutagenic and genotoxic properties
27 of VerA, yet several facets of its pronounced toxicity remain unexplored. In the present study,
28 we investigated early (6 h) transcriptomic changes induced by VerA in differentiated intestinal
29 cells in non-cytotoxic conditions (1 and 3 μM) and compared its effects to those of AFB1 at 1
30 μM . Our findings indicated that VerA led to substantial alterations in global gene expression
31 profiles, while AFB1 did not exhibit the same effect. As expected, both toxins caused
32 alterations in gene expression associated with well-known aspects of their toxicity, including
33 mutagenicity, genotoxicity, oxidative stress, and apoptosis. However, we also observed novel
34 features of VerA toxicity, including the ability to cause mitochondrial dysfunction and to
35 trigger a type-1 interferon response at least partially mediated by cGAS-STING. VerA also
36 induced changes in the expression of genes involved in the regulation of cell shape and
37 adhesion, transcription/translation as well as genes associated with tumor biology. Our results
38 offer new evidence of the high toxicity of VerA and underscore the importance of further
39 assessing the risk associated with its presence in food.

40 **Keywords:** Mycotoxins, Versicolorin A, Aflatoxin B1, transcriptome, intestine

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43 **Introduction**

44 Aflatoxins, produced by toxigenic *Aspergillus* fungi, pose a significant global food safety threat
45 (Bennett & Klich, 2003; Payros et al., 2021). These mycotoxins are widespread in hot and
46 humid regions, contaminating various agricultural products such as cereals, nuts, seeds, and
47 spices. Climate change projections indicate alarming increases in aflatoxin production in
48 European maize crops (Bailly et al., 2018; Battilani et al., 2016). Among aflatoxins, aflatoxin
49 B1 (AFB1) stands out as the most potent naturally occurring carcinogen (Schrenk et al., 2020).
50 Its mode of action depends on its dihydrobisfuran structure, which is activated *in vivo* by
51 cytochrome P450 enzymes and becomes a reactive mutagenic and genotoxic compound (Eaton
52 et al., 2010; Smela et al., 2001). To address this threat, current global regulations concerning
53 the presence of AFB1 in human food and animal feed authorize maximum levels of AFB1
54 ranging from 0.1 to 12 µg/kg depending on the product and its destination (European
55 Communities, 2023).

56 The biosynthesis pathway of AFB1 is well known and involves the production of different
57 bisfuranoid precursors that can accumulate in contaminated foodstuffs, including versicolorin
58 A (VerA) (Caceres et al., 2020; Jiang et al., 2019). Like AFB1, VerA is synthesized by
59 aflatoxigenic *Aspergillus* species. Additionally, non-aflatoxigenic *Aspergillus* species produce
60 VerA, explaining its detection at higher concentrations than AFB1 in certain food products
61 (Abdallah et al., 2017; Gauthier et al., 2020; Janić Hajnal et al., 2020). VerA is an emerging
62 mycotoxin and some its toxicological characteristics have already been elucidated, but VerA is
63 not yet subject to regulation. Like AFB1, VerA is highly mutagenic and genotoxic. Positive
64 mutagenic responses have been demonstrated in bacterial mutation assays, when the effects
65 were attributed to its metabolic activation in a potentially DNA-reactive form similar to that
66 produced by AFB1 (Al-Ayoubi et al., 2022, 2023). Using metabolically competent and non-
67 carcinogenic small intestinal cells (IPEC1) (Al-Ayoubi et al., 2023), we recently showed that a
68 short (6 h) exposure to concentrations of VerA as low as 0.03 µM caused chromosomal damage
69 and various pre-mutagenic lesions. Additional studies highlighted the greater complexity of
70 VerA toxicity compared to that of AFB1, driven by certain key factors. For example, exposure
71 to VerA induces higher levels of oxidative and replication stress in exposed cells than AFB1,
72 which emerge as potentially important contributors to its genotoxicity, and are linked to the
73 particular ability of VerA to induce DNA damage even in cells with poor metabolic activity
74 (Gauthier et al., 2020; Theumer et al., 2018). In acellular models, VerA was also identified as a

75 potent inducer of mitochondrial respiration uncoupling, warranting more research (Kawai et al.,
76 1983).

77 The present study aimed to understand the toxicity mechanisms of VerA in the intestine in order
78 to improve our understanding of VerA toxicity, since present data suggest that VerA may have
79 a more potent toxic effect than AFB1 and that the mechanisms underlying the toxicity of the
80 two toxins are not the same.

81

82 **2. Material and Methods**

83 **2.1. Toxins**

84 VerA was produced using an in-house method detailed in (Theumer et al., 2018). VerA was
85 isolated from an *Aspergillus parasiticus* strain (SRRC 0164) cultivated on wheat grain,
86 followed by purification using Ultimate 3000 high-performance liquid chromatography (HPLC;
87 Thermo Fisher Scientific, Courtaboeuf, France). The identity and purity of the VerA toxin were
88 determined by HPLC with a diode array detector and a Zorbax C18 analytical column, with
89 VerA peaks representing 97% of the signals (Theumer et al., 2018). AFB1 was purchased from
90 Sigma. Both toxins were dissolved in DMSO (Sigma) at 10 mM and stored at -20 °C until
91 further use.

93 **2.2. Cell culture and exposure conditions**

94 The non-tumorigenic and non-transformed intestinal cell line IPEC1 was chosen as a model
95 due to its competence in terms of cytochrome P450 and p53 status which made these cells
96 suitable for the study of aflatoxins and other pro-toxicants (Nossol et al., 2015). The IPEC1
97 cells were grown as described elsewhere (Al-Ayoubi et al., 2023). IPEC1 cells were
98 differentiated into monolayers using Transwell devices prior as previously described (Al-
99 Ayoubi et al., 2022) and exposed apically to 1 µM and 3 µM VerA, 1 µM AFB1, or to control
100 solvent (DMSO) for 6 hours. As described previously (Al-Ayoubi et al., 2023), these conditions
101 were not cytotoxic.

102 **2.3. Microarray and functional analysis of differentially expressed genes**

103 RNA isolation was performed as previously described (Pinton et al., 2010). Following
104 extraction, the concentration and quality of the RNA were assessed using a Dropsense 96
105 UV/VIS droplet reader (Trinean, Belgium) and an Agilent 2100 Bioanalyzer (Agilent
106 Technologies Inc., Santa Clara, CA, USA). The mean (\pm SD) RNA Integrity Number (RIN)
107 was 9.53 ± 0.36 . Gene expression profiling of the 16 samples (4 replicates per treatment group)
108 were conducted at the GeT-TRiX platform (GénoToul, Génomole Toulouse Midi-Pyrénées)
109 using Agilent Sureprint G3 Porcinet 60K_DEC2011 microarrays (8 × 60K, design 037880) in
110 accordance with the manufacturer's instructions, as described in (Pierron et al., 2022).
111 Microarray data and experimental details are available in NCBI's Gene Expression Omnibus
112 (Edgar et al., 2002) and are accessible through GEO Series accession number GSE267572
113 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE267572>).

114

115 **2.4. Real-time Quantitative PCR (RT-qPCR) analysis**

116 RT-qPCR assays were performed as described elsewhere (Hasuda et al., 2022). Primers (Table
117 S1, Supplementary data) were designed using PrimerQuest software (Integrated DNA
118 Technologies, San Diego, CA, USA) and were obtained from Sigma-Aldrich. Data analyses
119 were performed with the LinRegPCR freeware (version 2016) and normalized against different
120 reference genes (*HMBS*, *HPRT*, *TOP2B*).

121

122 **2.5. Protein Extraction and Immunoblotting**

123 Total protein extraction and immunoblotting were performed using methods detailed elsewhere
124 (Soler et al., 2020) using the primary antibodies listed in Table S2 and species-specific
125 fluorescent secondary antibodies (Biotium, Inc., Fremont, CA, USA). Target proteins were
126 visualized using a Fluorescent Imaging Scanner (Azure Biosystems, Dublin, CA, USA) and the
127 resulting images were digitized and quantified using Image Studio Lite Software v5.2 (Li-Cor
128 Biosciences, Lincoln, NE, USA). Band intensity values in each lane were normalized to their
129 respective total protein staining, which was performed using SYPRO™ Ruby protein blot stain
130 (Thermo Fisher Scientific) after transfer using ChemidDoc (SYPRO™ Ruby-stained
131 membranes; Bio-Rad).

132

133 **2.6. Evaluation of sirtuin activity in subcellular fractions**

134 Sirtuin deacetylase activity was assessed using a single reagent luminescence-based assay
135 (SIRT-Glo™, Promega). Protein extraction and subcellular fractionation (cytoplasmic and
136 nuclear protein fractions) were obtained using a commercially available kit (Subcellular Protein
137 Fractionation Kit, Thermo Scientific). NAD⁺ dependent histone deacetylase activity was then
138 measured in the presence of a histone deacetylase (HDAC) inhibitor, Trichostatin A (1 µg/mL,
139 Sigma), following the manufacturer's instructions using a luminescence plate reader (TECAN
140 Spark, Männedorf, Switzerland).

141 **2.7. Evaluation of mitochondrial dysfunction**

142 The impact of VerA on mitochondria was evaluated by comparing the ability of exposed cells
143 to produce ATP under non-cytotoxic conditions, both in the presence of glucose and when
144 glucose was replaced by D-galactose. Briefly, 10⁴ cells per well were seeded in white 96-well

145 plates and exposed to 1 μ M and 3 μ M VerA, for 6 h in the presence and absence of glucose,
146 which was subsequently replaced by D-galactose (Sigma).

147 The analysis was completed by evaluating cytotoxicity and caspase 3/7 activation using the
148 CytoToxFluor™ fluorescent assay (CytoTox-Fluor™, Promega) and the Caspase-Glo® 3/7
149 luminescent assay (Caspase-Glo, Promega), respectively. Following the manufacturer's
150 instructions, the assays were performed in cells ($5 \cdot 10^3$ cells/well) seeded in white 96-well plates
151 and exposed to the aforementioned concentrations of VerA for 6 hours.

152 The luminescence and fluorescence signals were recorded after exposure with a multimode
153 microplate reader (TECAN Spark). Three independent experiments with three technical
154 replicates were conducted for each condition to ensure the reliability of the results.

155

156 **2.8. Statistical analysis**

157 Microarray data were analyzed using R (R Core Team, 2018) and Bioconductor packages
158 (Huber et al. 2015) as described in GEO accession GSE267572. Raw data (median signal
159 intensity) were filtered, log₂ transformed and normalized using the quantile method (Bolstad et
160 al., 2003). A model was fitted using the limma lmFit function (Ritchie et al., 2015). Pair-wise
161 comparisons between biological conditions were applied using specific contrasts. A correction
162 for multiple testing was applied using the Benjamini-Hochberg procedure (BH, Benjamini &
163 Hochberg, 1995) to control the false discovery rate (FDR). Probes with FDR < 5% were
164 considered to be differentially expressed between conditions. The results were explored using
165 MATRIX APP (Lippi & Soubès, 2023, <https://matrix.toulouse.inrae.fr/>). Hierarchical
166 clustering was applied to the samples and the differentially expressed probes using 1-Pearson
167 correlation coefficient as distance and Ward's criterion for agglomeration and the results are
168 illustrated as a heat map. Functional analysis of the differentially expressed genes lists was
169 performed using the EnrichR web server (Chen et al., 2013; Kuleshov et al., 2016; Xie et al.,
170 2021) and Ingenuity Pathway Analysis tool (QIAGEN, Valencia, CA, USA). Only pathways
171 with a P value < 0.05 are shown here.

172 For cell-based assays, data from qPCR and immunoblotting tests are expressed as mean \pm
173 standard deviation (SD) of three independent experiments. The statistical analysis was
174 performed by a one-way Anova followed by Bonferroni's multiple comparisons tests using

175 GraphPad Prism version 9.0.2 (San Diego, California USA). A p-value < 0.05 was considered
176 statistically significant.

177

178 **3. Results and discussion**

179 **3.1 VerA, but not AFB1, induces marked changes in the gene expression profiles of** 180 **differentiated intestinal epithelial cells**

181 Exposure of intestinal cells to these conditions resulted in significant changes (FDR < 0.05;
182 fold-change > 1) in gene expression compared to vehicle (DMSO) controls. AFB1 induced
183 changes in 79 genes, while 1 μ M VerA and 3 μ M VerA induced changes in 3,261 and 5,211
184 genes, respectively (Fig. 1, A). Microarray results were confirmed by RT-qPCR analysis using
185 selected genes (*FLVCR2*, *SRM*, *MAPK2K6*, *SERPINB2* and *MX2*), and gene expression results
186 obtained using the two techniques were in agreement (Table S3). Our study found that AFB1
187 exposure had a low impact on the transcriptome of intestinal cells, in contrast to the high impact
188 of VerA. This observation aligns with previous research conducted on proliferating epithelial
189 intestinal Caco-2 cells (Gauthier et al., 2020). However, it's important to note that our study
190 identified fewer genes affected by VerA and AFB1 exposure compared to the aforementioned
191 study. This discrepancy may be attributed to the non-proliferative state of the monolayer cells,
192 as previously described for another mycotoxin (Hinze & Boucrot, 2018; Luo et al., 2021).

193 Principal component analysis (PCA) revealed that the concentration of VerA was the main
194 source of variance (Fig. 1, B). The Venn diagram in Figure 1A reveals significant similarity in
195 VerA-treated samples. Specifically, 2,788 genes were identified in samples treated with both 3
196 μ M and 1 μ M VerA, accounting for 85.5% of all genes regulated at a VerA concentration of 1
197 μ M. Additionally, 65 genes were found in both AFB1- and VerA-treated samples (at
198 concentrations of both 3 μ M and 1 μ M), representing 82.3% of all genes regulated at an AFB1
199 concentration of 1 μ M.

200 Next, double hierarchical clustering was performed to identify specific and shared expression
201 profiles for differentially expressed probes (FRD < 5%) identified in response to treatments.
202 The results are presented in the form of a heatmap (Fig. 1C). Three distinct gene clusters are
203 highlighted as alternative grey levels next to the dendrogram on the left based on the
204 relationships between their expression (Fig. 1 D). Cluster 1 included 2,695 genes that were
205 down-regulated in all VerA-treated cells compared to in controls, although AFB1 had relatively

206 weaker effects and the responses to VerA were dose dependent. Functional analysis (Table S4)
207 revealed that the main regulated pathways of this cluster (i.e. those showing the most significant
208 enrichment) were related to cadherin-mediated cell adhesion and changes in shape and their
209 related signaling, as well as RNA processing. The predicted upstream regulators of the genes
210 in this cluster were mainly *TP53* and *TGFBI*. The ‘top tox’ list (set of molecules that are known
211 to be involved in a particular type of toxicity) was primarily related to cell death. Notably, the
212 list of ‘top analysis-ready molecules’ (an output list showing the genes with the strongest
213 regulation; Table S5) mainly included genes involved in regulating cell adhesion and shape-
214 related functions (*DKK1*, *SPRED1*, *TUBB6*, *FGD6*), and p53-related signaling (*PPP1R13B*,
215 *PRRG1*, *NAVI*).

216
217 Cluster 2 consisted of 726 genes that were upregulated at a similar magnitude in all treated
218 cells. The differentially expressed genes in this cluster were mainly related to interferon
219 signaling, methionine and L-cysteine degradation, mesenchymal cell differentiation mediated
220 by *RUNX1* and caspase activation (Table S6). The primarily predicted upstream regulators of
221 these processes were identified as poly rI:rC RNA and *RNASEH2A*. In the context of toxicity,
222 the ‘top tox’ molecular set was primarily associated with oxidative stress. The genes that
223 showed the strongest regulation in cluster 2 (Table S7) included several involved in processes
224 related to tumorigenesis (*TGFA*, *RDH16*, *UTRN*), transcription regulation (*KLF3*), DNA repair
225 (*AHDCl*) and apoptosis (*CASP5*).

226
227 Cluster 3 included 1,997 genes upregulated in all treated samples compared to in the control
228 group but the magnitude of change in gene expression was only high in cells treated with VerA,
229 mostly at a concentration of 3 μ M. Functional analysis based on the most significant p-values,
230 revealed that the main enriched pathways in this dataset were the sirtuin signaling pathway,
231 mitochondrial dysfunction, interferon signaling, and processes related to DNA damage and
232 repair (Table S8). *TP53* and *HNF4A* were identified as main predicted upstream regulator in
233 many of these processes. Additionally, the enriched GO molecular functions mainly included
234 oxidized DNA binding and glutathione transferase activity. The genes showing the strongest
235 regulation in cluster 3 (Table S9) are mainly known for their critical functions in tumor biology
236 (*NR3C2*, *SNTB1*, *ABLIM1*, *PPP1R9A*, *MBP* and *GHR*). The list was completed by chaperones
237 and genes involved in immune signaling (*CCDC30*, *FLVCR2* and *SLC7A7*).

238

239 **3.2 Both AFB1 and VerA induce overexpression of DNA damage response-related genes:** 240 **mutagenicity as a common mechanism of action**

241 Almost all the genes significantly regulated by AFB1 were also regulated by VerA at both
242 concentrations tested (65 genes; Fig.1, A) although the fold-changes in gene expression were
243 more pronounced at a concentration of 3 μ M VerA than at 1 μ M VerA and that of AFB1 for
244 these genes in all functional categories. The main function of these genes was shown to be
245 associated with DNA repair, cell cycle control and apoptosis. This result reflects the common
246 toxicity of AFB1 and VerA as genotoxins. In our previous works, we demonstrated that VerA
247 is likely to be biotransformed into a mutagenic derivative, which is known to be the main
248 mechanism of action of AFB1 (Al-Ayoubi et al., 2022). Indeed, the signaling pathway ‘diseases
249 of mismatched repair’ that is known to be activated in the presence of mutagenic agents, is
250 overrepresented in our dataset. Further, both toxins can cause oxidative DNA damage (Al-
251 Ayoubi et al., 2023). Our gene expression results consequently reflect the fact that both toxins
252 can activate a DNA damage response (DDR) triggered by their mutagenicity.

253 According to our data, the DDR induced by both toxins seems to be mediated by ataxia
254 telangiectasia-mutated - breast cancer 1 (ATM-BRCA1) and the tumor suppressor protein p53
255 pathways, potentially involving upstream regulators with roles already described during the cell
256 response to AFB1, such as p53, HNF-4 α (Marrone et al., 2016) and RNase H2. In response to
257 DNA damage, p53 binds DNA and mediates transcriptional activation of genes whose products
258 trigger cell-cycle arrest, DNA repair and/or apoptosis (Riley et al., 2008). The essential role of
259 this tumor suppressor protein in the alleviation of the toxicity of AFB1 is known, and a mutation
260 in codon 249 of the *TP53* gene is tightly linked with the development of hepatocarcinoma in
261 individuals exposed to AFB1 (Hussain et al., 2007). While previous studies suggested that p53
262 is not essential for cell cycle control and apoptosis induction in intestinal cells exposed to VerA
263 (Gauthier et al., 2020), we observed that 53BP1 is an important mediator of VerA-induced
264 DDR, as it is recruited in IPEC1 cells exposed to VerA (Al-Ayoubi et al., 2023). According to
265 the literature, some post-translational modifications are important regulators of p53 activity
266 (Meek & Anderson, 2009) for example, Ser15 phosphorylation and Lys382 acetylation.
267 Previous studies showed that AFB1 induces significantly higher levels of p53-Ser15
268 phosphorylation than VerA in Caco-2 and HepG2 cells (Budin et al., 2021; Gauthier et al.,

269 2020), whereas in our study, only VerA induced a significant increase of Ser15-phosphorylated
270 p53 levels in IPEC1 cells (at the highest concentration), while AFB1 did not (Fig. 2). What is
271 more, only VerA induced changes in gene expression in many p53-responsive genes including
272 *PPP1R13B*, *PRRG1*, *NAV1*, *CD82*, *PIK3R3*, *PIK3C2B* and *SESNI*, suggesting that VerA
273 activates p53 responses in exposed IPEC1 cells. Indeed, our results agree with previous findings
274 indicating that wild-type p53 dynamics varies substantially between cell lines (Stewart-
275 Ornstein & Lahav, 2017).

276 In addition to phosphorylation, Lys382 acetylation is a p53 transactivation pathway that
277 enhances the sequence-specific DNA-binding activity of p53 in response to DNA damage, and
278 is controlled by several NAD⁺-dependent deacetylases, including sirtuin 1 (SIRT1).
279 Interestingly, we observed significant overrepresentation of the sirtuin signaling pathway and,
280 in general, of genes exhibiting deacetylase activity in VerA-exposed cells. We examined the
281 cytoplasmic and nuclear sirtuin-dependent deacetylase activity in IPEC1 cells exposed to 3 μM
282 VerA and only detected a significant increase in nuclear sirtuin-dependent deacetylase activity
283 (Fig. 3, A). Protein abundance levels of the nuclear sirtuin SIRT1 (Fig 3, B) were also
284 significantly increased in the presence of VerA (at both 3 μM and 1 μM), as well as in the
285 presence of AFB1 (1 μM). In parallel with these results, we observed a concomitant VerA-
286 dependent regulation of the expression of downstream genes within the SIRT1 pathway, such
287 as *PPARGC-1*, *PPARG* and *FXR* (p-value < 0.05). These data suggest that sirtuins, particularly
288 SIRT1, play a role in the toxicity of VerA. Since SIRT1 mediates the acetylation of p53 and
289 this posttranslational modification is important for the activity of p53 (Ren et al., 2019), we
290 investigated whether VerA induces changes in Lys382-acetylation of p53. However, our
291 findings revealed a non-significant accumulation of acetylated p53 in exposed cells (Fig. 2).
292 More research is thus needed to understand the role of SIRT1 in VerA toxicity, which may
293 involve transcriptional regulation of p53/histone acetylation (Zhang & Kraus, 2010), or be
294 associated with mitochondrial toxicity (discussed below) and apoptosis induction (Tang, 2016).
295 Our results confirm that DDR is initiated by both AFB1 and VerA, confirming a common
296 mechanism of action in these structurally related compounds, based on their mutagenicity.
297 While this requires further studies, our data also suggest that, contrary to previously published
298 results, p53 may be also an important mediator of DDR for VerA, as it is for AFB1.

299

300 **3.3 VerA: an inhibitor of transcription?**

301 Previous results indicated that VerA genotoxicity involves more mechanisms than that of
302 AFB1, mainly the induction of a higher level of replication stress (Al-Ayoubi et al., 2023;
303 Gauthier et al., 2020). Replication stress may explain the ability of VerA to induce genotoxicity
304 in cell lines that lack metabolic activity, in contrast to AFB1 (Theumer et al., 2018). The results
305 of functional analysis of our data are in line with this hypothesis and provide additional clues
306 to how VerA could induce replication stress. We found an enrichment of molecular functions
307 related to the biology of DNA and RNA in VerA-regulated genes. The RNA metabolism
308 pathway, along with molecular functions related to RNA and snoRNA binding were
309 significantly overrepresented. When we extracted the list of genes associated with the GO
310 “RNA binding” from our dataset (Table 1), we observed that the expression of these genes is
311 only regulated by VerA. Most of these genes, which are essential for correct transcription and
312 translation, were significantly down-regulated, while the expression of the topoisomerases 1
313 and 2A was significantly up-regulated. Previous results (Gauthier et al., 2020) indicated that
314 VerA exposure leads to the significant accumulation of small nucleolar H/ACA RNA (SNORA)
315 and C/D box RNA (SNORD) genes. Taken together, these results suggest that VerA may
316 disrupt DNA/RNA biology, which at least partially explains the known activation of an early
317 ATR response, increased FANCD2 nuclear accumulation and the overall high replication stress
318 induced by VerA (Al-Ayoubi et al., 2023; Gauthier et al., 2020). Additional studies are needed
319 to understand the mechanism by which VerA interferes with transcription and/or translation.

320

321 **3.4 VerA induces higher oxidative stress than AFB1 depending on changes in gene** 322 **expression**

323 Oxidative stress was one of the ‘top tox’ to emerge from our functional analysis of genes in
324 cluster 2, while oxidized DNA binding was identified as an enriched molecular function of
325 genes in cluster 3. Additionally, two overrepresented pathways in cluster 2 were associated with
326 enhanced degradation of methionine and its intermediary metabolite, L-cysteine. Since
327 methionine plays a significant role as a scavenger of reactive oxygen species (ROS) (Shen Luo

328 & Levine, 2009), our results suggest toxin-mediated oxidative stress and, as a consequence,
329 enhancement of methionine degradation. Oxidative stress occurs in AFB1-exposed cells as a
330 consequence of ROS accumulation during the biotransformation of AFB1 into its reactive
331 epoxide form (Kozieł et al., 2021). This leads to activation of the antioxidant Nrf2/ARE
332 pathway and depletion of the methionine cell pool. It is known that dietary methionine
333 supplementation mitigates the toxicity of AFB1 by modulating an Nrf2- mediated antioxidant
334 pathway (Elwan et al., 2021). To check whether the predicted increased degradation of
335 methionine was associated with changes in the expression of effector genes in the Nrf2 response
336 (Table 2), we monitored expression of key genes in our dataset. Our analysis revealed that
337 exposure to VerA was significantly associated with upregulation of *CAT*, *GPX8*, *NQO1* and
338 *GCLM* and with downregulation of *SOD2*, *GPX2*, *HMOX1*, *GCLC* and *TXN*. In contrast,
339 exposure to AFB1 only caused significant downregulation of the intestinal glutathione
340 peroxidase *GPX2*. These results suggest the presence of oxidative stress and the activation of
341 Nrf2 responses in VerA- but not in AFB1-treated cells. This is in line with the results of
342 previous studies showing that VerA induces higher oxidative stress than AFB1 in intestinal
343 cells, characterized by a significant accumulation of ROS, including hydrogen peroxide and
344 superoxide anion (Gauthier et al., 2020).

345

346 **3.5 VerA, but not AFB1, induces mitochondrial toxicity and an IFN-I response mediated,** 347 **at least partly, by cGAS-STING**

348 Functional analysis indicated an increase in oxidoreductase activity and signs of mitochondrial
349 dysfunction, particularly among the genes in cluster 3. Only VerA, mainly at the highest
350 concentration, but not AFB1, regulated these genes associated with the mitochondrial
351 dysfunction, as identified by the functional analysis. We observed that, among the genes
352 regulated across all mitochondrial respiratory complexes, those in complex IV underwent a
353 more pronounced change in their fold-change ratio (Fig. S1). These data suggest that VerA is
354 toxic to the mitochondria, which was already suggested in early studies using acellular models
355 (Kawai et al., 1983). In fact, mycotoxins with a hydroxyl group at the β -position of their
356 anthraquinone nucleus, like VerA, are known to have a strong uncoupling activity in
357 mitochondrial respiration (Kawai et al., 1983), which is responsible for their high cytotoxicity.

358 Reversal potential analysis, using planar lipid bilayer membranes, showed that VerA facilitates
359 proton permeability and increases conductance, which partly explain its uncoupling effect on
360 mitochondrial respiration (Muto et al., 1997).

361 The mitotoxicity of VerA was confirmed in our cell model since a significant drop in ATP
362 levels was observed when exposed IPEC1 cells were forced to only use oxidative
363 phosphorylation to produce ATP. This effect was associated with significant activation of
364 caspase 3/7, effectors of the intrinsic apoptotic pathway. Although cytotoxicity did not increase
365 significantly, cells exposed to 3 μ M VerA showed more pronounced mitotoxicity and oxidative
366 and pro-apoptotic processes than cells exposed to 1 μ M VerA (Fig. 4). Moreover, VerA (but
367 not AFB1) promoted changes in genes related with mitophagy such as *SQSTM1* and *HSPD1*.
368 HIF-1, a protein known to respond to mitochondrial stress (Huang et al., 2023), accumulated in
369 cells exposed to VerA, while BNIP3L, a protein degraded together with mitochondria during
370 mitophagy, was significantly depleted (Fig. S3). All these findings provide strong evidence that
371 VerA has mitotoxic properties, calling for characterization of its impact on cell respiration,
372 metabolism and mitochondrial biogenesis.

373
374 While we cannot rule out involvement of ATM-dependent NF κ B activation in response to
375 genomic DNA damage, the modulation of genes associated with activation of interferon (IFN)
376 signaling responses observed in our dataset (Table S10) may also be attributed to VerA-specific
377 mitochondrial dysfunction. Indeed, exposure to VerA induced the over-expression of IFN- β 1,
378 the IFN receptor IFNGR2, the transcriptional regulators of type I IFN-dependent immune
379 responses, and the interferon-inducible gene *IRF3* as well as the IFN-inducible genes *GBP2*,
380 *MX2*, *OAS1* and *OAS2*. The interferon-I pathway can be activated by the accumulation of ROS
381 and mitochondrial ROS (MitoSOX) during mitochondrial toxicity. As mentioned earlier, VerA
382 is known to induce significantly higher oxidative stress than AFB1 (Gauthier et al., 2020), and
383 exposure to 3 μ M VerA was able to increase the levels of mitochondrial superoxide in IPEC1
384 cells after 24 hours of incubation (Fig. 4, E). In addition, mitochondrial damage can lead to the
385 release of mitochondrial DNA (mtDNA) in the cytoplasm, which is sensed by the cyclic GMP-
386 AMP synthase-stimulator of interferon response cGAMP interactor 1 (cGAS-STING) pathway,
387 a potent inducer of type I IFN responses (West et al., 2015). Our data strongly suggest that
388 cGAS-STING is activated upon exposure to VerA, as we observed significant upregulation of
389 STING, as well as the effector genes of this pathway *TBK1*, *PTEN*, *PIAS1* and *STAT1/2* (Table
390 S10). Along with these changes, cells exposed to VerA also accumulated significant quantities

391 of phospho-STAT1 (Fig. S3). In line with these findings, our study indicates that similar pro-
392 inflammatory signaling pathways engaged by mtDNA include the NLRP3 inflammasome and
393 NF- κ B pathways following exposure to VerA. Signs of activation of both pathways by VerA
394 were observed, such as higher gene expression of CASP1 and IL1B (Table S10), as well as
395 accumulation of IKB α protein in exposed cells (Fig. S3). Overall, VerA is toxic to
396 mitochondria, inducing a cascade of pro-inflammatory signals, which highlights the importance
397 of understanding the relevance of these events, as mtDNA-induced responses are implicated in
398 the promotion of autoimmune, metabolic and degenerative diseases.

399 **3.6 Is VerA cancerogenic? Evidence from gene expression analysis**

400 As discussed above, VerA is mutagenic and genotoxic, and shares some features of toxicity
401 with AFB1. Yet, while AFB1 is known to be a cancerogenic compound, this is not the case for
402 VerA. However, the present dataset has provided information concerning the phenotypic
403 changes that cells undergo after exposure to VerA that suggest a cancerogenic potential.

404 A group of genes in cluster 1 is functionally involved in the remodeling of epithelial adherent
405 junctions, integrin signaling, and actin cytoskeleton signaling. These genes were significantly
406 downregulated (adjusted $p < 0.05$) in cells exposed to VerA in a concentration-dependent
407 manner, but not in cells exposed to AFB1 (Table S5, Fig.S2). These changes in gene expression
408 suggest that epithelial cells exposed to VerA undergo phenotypical changes related to loss of
409 adhesion, alterations to cell shape and to cell junctions. Functional analysis predicted that the
410 upstream regulators of these changes would transform growth factor-beta (TGF- β ; TableS4)
411 and tumor protein p53 (*TP53*; Table S4). TGF- β is a potent growth suppressor of epithelial cells
412 and mediates cell differentiation of intestinal epithelial cells (Morikawa et al., 2016), and the
413 mutation of *TP53* has been associated with, among other things, increased adhesion ability (Lee
414 et al., 2015). In line with these findings, many genes in this cluster were found to be involved
415 in cell growth, death control and motility (Table S5). The 10 top most regulated genes in this
416 cluster code for proteins present in the cytoskeleton (*TUBB6*, *FDG6*), nucleosome (*PROB1*) or
417 chaperone protein complexes (*SDF2L1*) or are else involved in p53 signaling (*PPP1R13B*,
418 *PRRG1*, *NAVI*, *EEPD1*) or in the regulation of epithelial-mesenchymal transition (EMT)
419 initiation (*DKK1*, *SPRED1*). In line with these results, genes related to RUNX1-mediated
420 signaling (RUNX1 regulates TGF- β induced EMT; (Lu et al., 2020)) were overrepresented in
421 cluster 2, and many genes involved in EMT initiation and progression, such as *TGFA*, *SNTB1*,
422 *ABLIM* and *PPP1R9A*, were among the most regulated genes in clusters 2 and 3. EMT is a

423 process in which polarized epithelial cells lose their epithelial phenotype and adopt a
424 mesenchymal cell phenotype, including, among other changes, increased resistance to
425 apoptosis, which is often associated with malignant transformation of epithelial cells (Koen &
426 Collier, 2009).

427 Despite all the evidence for the induction of an EMT in differentiated intestinal cells in our
428 dataset, we were not able to confirm modulation of CTNN β and E-cadherin at the protein level.
429 Furthermore, the abundance of other proteins, including the actin-regulating protein cofilin and
430 the mesenchymal phenotype marker vimentin, did not change (Fig. S3). Because the short
431 exposure time (6 h) may be insufficient to induce transition to a mesenchymal phenotype,
432 studies with adjusted exposure conditions (non-cytotoxic concentrations, longer exposure) are
433 now needed to determine whether VerA reduces the presence of membrane E-cadherin, causes
434 trans-nucleation of β -catenin or promotes changes in the actin cytoskeleton related to an EMT
435 transition, plus to identify the role of TGF- β in this phenomenon.

436 Interestingly, several of the genes most regulated by VerA were shown to be involved in tumor
437 biology. The list includes different tumor suppressing genes such as *RDH16*, strongly regulated
438 in hepatocellular cancer (Zhu et al., 2020), *UTRN*, which is mutated in a number of tumors (Y.
439 Li et al., 2007) and *NFIB*, also known to have tumor suppressive functions in many
440 malignancies. Other genes known to be involved in colorectal tumorigenesis such as *NR3C2*,
441 which is reported to function as a tumor suppressor (J. Li & Xu, 2022), *GHR*, which is
442 frequently expressed in human colorectal cancers (Yang et al., 2004) and *ABLIM1*, which
443 promotes growth and metastasis of colorectal cancer (He et al., 2024), were all modulated. This
444 list is completed by *SNTB1*, which plays a crucial role in the stemness maintenance of cancer
445 cells (Liang et al., 2021) and *PPP1R9A*, an imprinted gene, whose imprinting loss is linked
446 with the development of different epithelial types of cancer (Hsu et al., 2016).

447 Overall, our results support the hypothesis that exposure to VerA is associated with the
448 induction of cell malignancy in intestinal cells, underlining the importance of investigating the
449 carcinogenic capacities of VerA.

450

451 **4. Conclusion**

452 Our study reveals new insights into VerA toxicity, confirming its greater toxicity to intestinal
453 cells compared to AFB1. While VerA-induced transcriptome changes mirror those associated
454 with DNA damage response, oxidative stress, and apoptosis seen with AFB1, VerA affects a

455 larger number of genes and exhibits unique toxic aspects. Our data suggest that VerA's
456 genotoxicity may involve replication stress due to RNA/DNA biology disruption. Additionally,
457 VerA shows mitotoxicity in intestinal cells, potentially linked to interferon-I immune responses
458 and inflammatory cascades. Dysregulation of genes related to a carcinogenic cell phenotype
459 was also observed. These findings contribute valuable toxicity data for assessing VerA
460 exposure risk.

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465 5. References

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Figures:

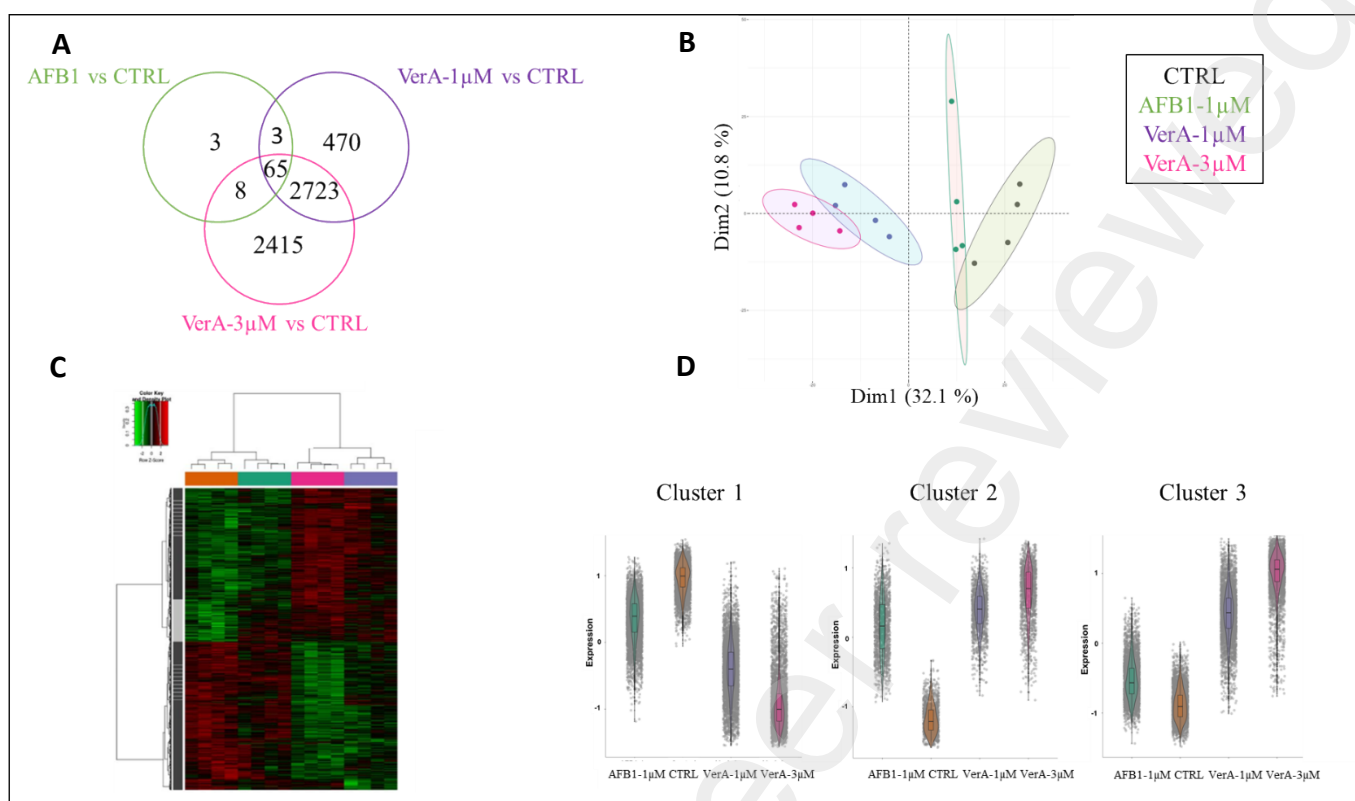


Figure 1. Gene expression profile of intestinal cells exposed to AFB1 (1 μ M), VerA (1 μ M), VerA (3 μ M)

(A) Venn diagram representing the differentially expressed genes (Adjusted p -value < 0.05) in cells exposed to 1 μ M AFB1 (in green), 1 μ M VerA (in purple) and 3 μ M VerA (in pink) compared to solvent control cells. (B) Principal component analysis of normalized expression signals on all samples: 1 μ M AFB1 (green), 1 μ M VerA (purple) and 3 μ M VerA (pink) and solvent control DMSO (black). (C) Heat map representing differentially expressed genes exposed to AFB1 (1 μ M), VerA (1 μ M), VerA (3 μ M) compared to control (DMSO, adjusted p -value < 0.05). Individual gene expression is represented in the heatmap, and the hierarchical clustering was obtained from individual expression values using Pearson correlation coefficient as distance and Ward's criterion for agglomeration. Red and green indicate values above and below the mean averaged centred and scaled expression values (Z-score), respectively. Black indicates values close to the mean. According to the probe clustering (left panel), 3 gene clusters showed specific gene expression profiles. (D) Violin plots with strip charts representing expression profiles of the three clusters identified by the clustering of differentially expressed probes illustrated in the heat map in (C). The average expression signal (z-score) of the probes is plotted for each of the four conditions: 1 μ M AFB1 (green), 1 μ M VerA (purple) and 3 μ M VerA (pink) and solvent control DMSO (orange) cells.

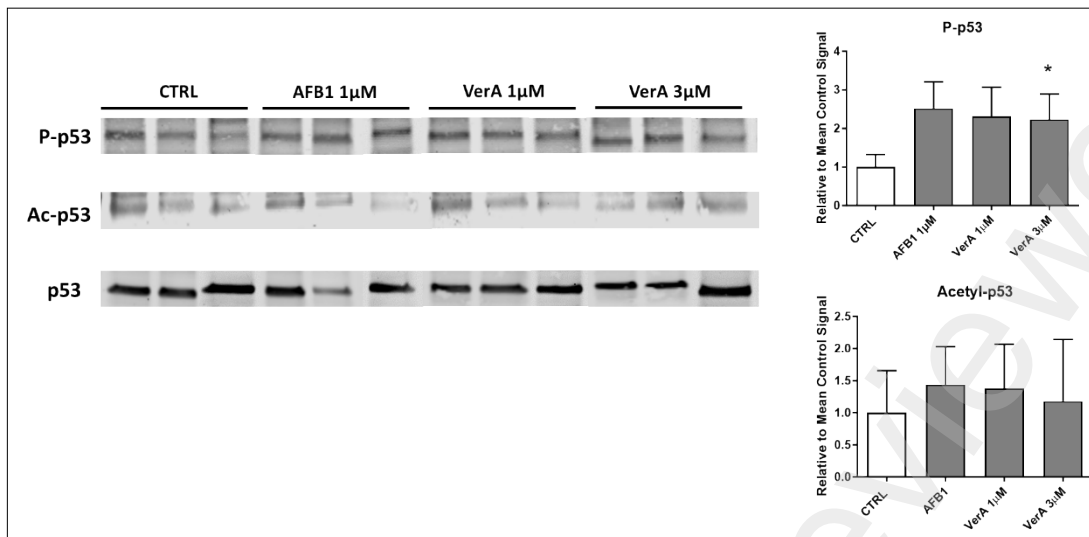


Figure 2. Immunoblotting analysis of phospho-p53 and acetyl-p53 in intestinal cells exposed to AFB1 (1 μ M), VerA (1 μ M), VerA (3 μ M) or not (CTRL). (A) Representative blots of 3 paired samples of phospho-p53, acetyl-p53 and p53 (B) Relative quantification of normalized signal (arbitrary units). Values of phospho-p53 and acetyl-p53 are relative to those of p53. Values are means with standard errors of the mean of the control represented by vertical bars ($n = 3$). Asterisks indicate statistical differences (* $p < 0.05$).

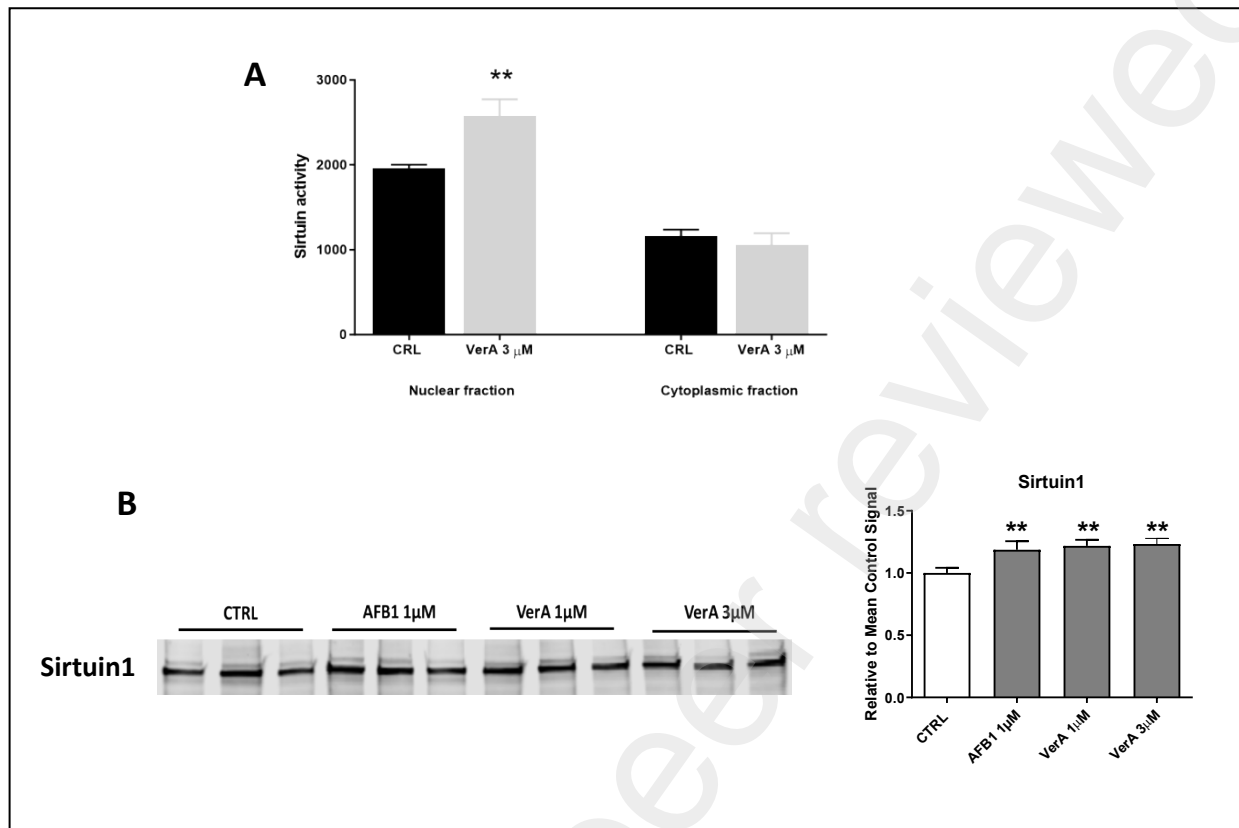


Figure 3. Effects of VerA on the sirtuin signaling pathway in intestinal cells. (A) Deacetylase activity of sirtuins in subcellular fractions of intestinal cells exposed to 3 μ M VerA for 6 h. Values are means with standard errors of the mean represented by vertical bars ($n = 3$). Asterisks indicate a significant difference (** $p < 0.01$). (B) Immunoblotting analysis of sirtuin 1 in intestinal cells exposed to AFB1 (1 μ M), VerA (1 μ M), VerA (3 μ M) or not (CTRL). (B, Left) Representative blots of 3 paired samples of sirtuin 1 (B, Right) Relative quantification of normalized signal (arbitrary units). Values are means with standard errors of the mean of the control represented by vertical bars ($n = 3$). Asterisks indicate statistical differences (** $p < 0.01$).

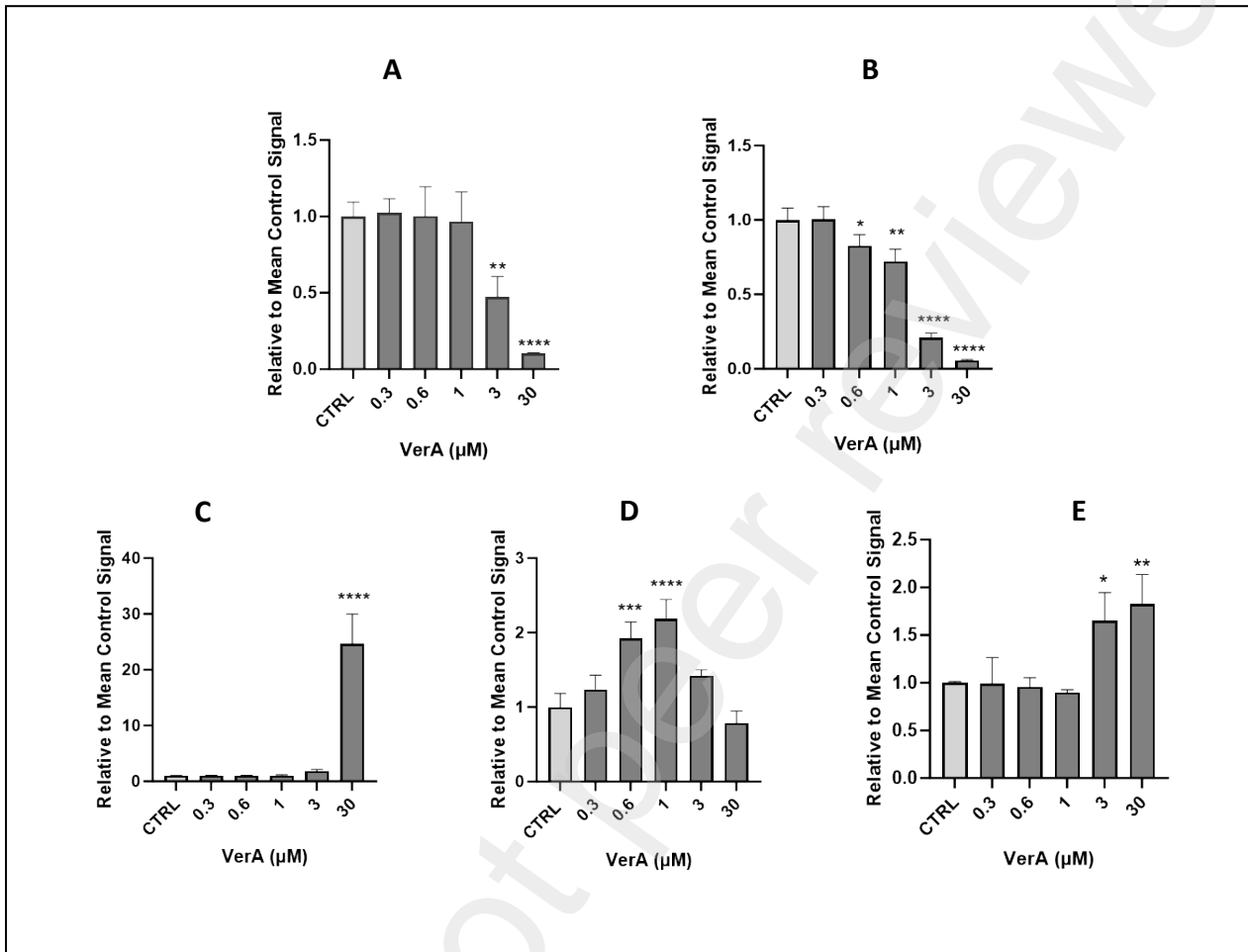


Figure 4. Evaluation of the mitochondrial toxicity of VerA in intestinal cells. Measurement of ATP levels in intestinal cells exposed to different doses of VerA for 6 h (A) in the presence of glucose or (B) in the presence of galactose. Evaluation of (C) cytotoxicity, (D) caspase 3/7 activation and (E) mitochondrial superoxide levels. Values are means with standard errors relative to the mean of the control ($n = 3$). Asterisks indicate statistical differences (* $p < .05$; ** $p < .01$, *** $p < .001$, **** $p < .0001$).

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Tables:

Table 1. Differentially expressed genes in cluster 1 associated with the GO molecular function “RNA binding” in intestinal cells exposed to AFB1 (1 μ M) and VerA (1 μ M / 3 μ M) compared to solvent control DMSO. Adjusted P.Values < 0.05 are highlighted in grey.

| Gene name | Genes associated with the GO RNA binding molecular function | | | | | | Function |
|-----------------------------|---|----------------|----------------|-------------------------|----------------|----------------|--|
| | adj.P.Val | | | Fold Change vs. Control | | | |
| | VerA 1 μ M | VerA 3 μ M | AFB1 1 μ M | VerA 1 μ M | VerA 3 μ M | AFB1 1 μ M | |
| <i>Down-regulated genes</i> | | | | | | | |
| <i>SRPK1</i> | 4.1E-03 | 9.1E-06 | 0.27 | 0.69 | 0.47 | 0.83 | Regulation of constitutive and alternative splicing |
| <i>EIF4G1</i> | 0.67 | 6.2E-03 | 0.54 | 0.96 | 0.8 | 0.92 | Essential for transcription |
| <i>DHX9</i> | 3.7E-02 | 0.14 | 0.2 | 0.77 | 0.85 | 0.79 | Involved in DNA replication, transcriptional activation, post-transcriptional RNA regulation, mRNA translation and RNA-mediated gene silencing |
| <i>NME1</i> | 3.6E-02 | 9.7E-03 | 0.59 | 0.85 | 0.83 | 0.93 | Involved in cell proliferation, differentiation and development, signal transduction, G protein-coupled receptor endocytosis, and gene expression. |
| <i>EIF4G2</i> | 0.12 | 1.2E-02 | 0.96 | 0.81 | 0.71 | 0.98 | General repressor of translation by forming translationally inactive complexes |
| <i>TSR1</i> | 2.4E-02 | 3.5E-04 | 0.95 | 0.82 | 0.65 | 0.98 | Involved in endonucleolytic cleavage of tricistronic rRNA transcript and maturation of SSU-rRNA from tricistronic rRNA transcript |
| <i>NOP56</i> | 5.3E-04 | 4.0E-08 | 0.95 | 0.78 | 0.57 | 0.99 | Core component of box C/D small nucleolar ribonucleoprotein (snoRNP) particles |
| <i>RAN</i> | 0.46 | 4.1E-04 | 0.99 | 0.95 | 0.79 | 1.00 | Favors translocation of RNA and proteins through the nuclear pore complex. Involved in control of DNA synthesis and cell cycle progression |
| <i>BMS1</i> | 8.8E-03 | 2.6E-05 | 0.81 | 0.87 | 0.77 | 0.97 | Processing of Capped Intron-Containing Pre-mRNA |
| <i>NOPI4</i> | 1.3E-02 | 8.8E-05 | 0.65 | 0.84 | 0.74 | 0.94 | Nucleolar processing of pre-18S ribosomal RNA |

| | | | | | | | |
|---------------------------|---------|---------|------|------|------|------|--|
| <i>GTPBP4</i> | 1.5E-03 | 1.7E-06 | 0.84 | 0.84 | 0.75 | 0.98 | Involved in the biogenesis of the 60S ribosomal subunit |
| <i>Up-regulated genes</i> | | | | | | | |
| <i>TOP1</i> | 9.0E-03 | 1.3E-04 | 0.71 | 1.15 | 1.22 | 1.05 | Controls and alters the topologic states of DNA during transcription |
| <i>TOP2B</i> | 8.9E-02 | 4.4E-04 | 0.86 | 1.10 | 1.20 | 1.03 | Controls and alters the topologic states of DNA during transcription |

Table 2. Differentially expressed genes from the Nrf2/ARE signaling pathway in intestinal cells exposed to AFB1 (1 μ M) and VerA (1 μ M / 3 μ M) compared to solvent control DMSO. Adjusted P.Values < 0.05 are highlighted in grey.

| Gene name | Genes involved in Nrf2/ARE response | | | | | |
|---------------|-------------------------------------|-------------------|-------------------|-------------------------|-------------------|-------------------|
| | adj.P.Val | | | Fold Change vs. Control | | |
| | VerA 1 μ M | VerA 3 μ M | AFB1 1 μ M | VerA 1 μ M | VerA 3 μ M | AFB1 1 μ M |
| <i>NFE2L2</i> | 0.10 | 0.42 | 0.33 | 1.17 | 1.08 | 1.15 |
| <i>KEAP1</i> | 0.99 | 0.94 | 0.97 | 1.00 | 0.99 | 1.01 |
| <i>CAT</i> | 3.73E-03 | 1.02E-03 | 0.52 | 1.27 | 1.29 | 1.10 |
| <i>GSS</i> | 0.76 | 0.55 | 0.90 | 0.96 | 0.94 | 0.96 |
| <i>SOD1</i> | 0.52 | 0.18 | 0.10 | 1.04 | 1.07 | 1.00 |
| <i>SOD2</i> | 0.13E-02 | 1.52E-04 | 0.15 | 0.74 | 0.72 | 0.83 |
| <i>GPX1</i> | 0.98 | 0.42 | 0.96 | 1.00 | 1.04 | 0.99 |
| <i>GPX1</i> | 0.15 | 2.81E-02 | 0.93 | 1.07 | 1.09 | 1.01 |
| <i>GPX2</i> | 3.33E-04 | 2.99E-06 | 2.74E-02 | 0.76 | 0.70 | 0.77 |
| <i>GPX3</i> | 3.13E-02 | 0.32 | 0.53 | 0.84 | 0.92 | 0.91 |
| <i>GPX4</i> | 0.69 | 0.27 | 0.82 | 1.02 | 1.05 | 0.97 |
| <i>GPX6</i> | 0.62 | 0.19 | 0.92 | 0.94 | 0.87 | 1.04 |
| <i>GPX8</i> | 7.99E-02 | 4.65E-03 | 0.95 | 1.12 | 1.19 | 1.01 |
| <i>NQO1</i> | 7.93E-06 | 3.97E-05 | 0.79 | 1.34 | 1.27 | 0.96 |
| <i>HMXO1</i> | 0.50 | 3.61E-03 | 0.47 | 0.95 | 0.86 | 0.93 |
| <i>GCLC</i> | 0.32 | 2.5 | 0.79 | 0.91 | 0.84 | 0.94 |
| <i>GCLM</i> | 0.63E-04 | 4.95E-06 | 0.82 | 1.19 | 1.32 | 1.03 |
| <i>TXN</i> | 0.135 | 5.99E-03 | 0.92 | 0.92 | 0.87 | 0.98 |