1 Zika virus infection induces synthesis of Digoxin in
2 glioblastoma cells

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9 **ABSTRACT**

10 Recently, microcephaly cases have increased in Americas and have been matter of concern due to Zika

11 virus (ZIKV) recent outbreak. Previous studies have shown that ZIKV-infected progenitor neuronal

12 cells present morphological abnormalities and increased rates of cell death, which may be indicators of

13 microcephaly causes. As recent studies indicate Zika virus' tropism for brain cells, how would a

14 glioblastoma (GBM) lineage behave under ZIKV infection, considering GBM the most common and

15 malignant brain tumor in adults, presenting extreme chemoresistance and high morbidity and mortality

16 rates? The current trend of using genetically engineered oncolytic pathogens as a safe way to eliminate

17 tumors is under development, with trials already in course. Therefore, the present study evaluated the

18 possible oncolytic effects and metabolomic alterations of Zika virus infection at human malignant

19 M059J glioblastoma cells. Microscopic evaluation was performed using optical microscopy, which

20 showed cytopathic effects induced by ZIKV at GBM cells. For the metabolomics study, both control

21 and infected cell cultures were submitted to MALDI-MSI analysis. Mass spectrometry data were

22 submitted to PLS-DA statistical analysis, and distinct biomarkers were elected for each infected

23 groups. This study brings light to unexpectedly induced metabolic changes, as endogenous Digoxin as

24 important biomarker for ZIKV-GBM group, associated with cytopathic effects induced by viral

25 infection. These results evidences that genetically engineered ZIKV might be a potential new strategy

26 for neural cancer management through the induction of endogenous digoxin synthesis in glioblastoma

27 cells.

28 **Keywords:** Zika virus, Glioblastoma, Oncolytic virus, Cancer management, Digoxin, Cardiac

29 glycoside.

INTRODUCTION

The recent outbreak of Zika virus (ZIKV) in Brazil and its outspread through the Americas has brought much concern on the effect that the infection might have on the long term [1]. Zika virus belongs to *Flaviviridae* family and is classified as an arbovirus, a descriptive term for RNA viruses transmitted by arthropods, remarkably mosquitoes of the genus *Aedes* [2]. Although ZIKV infection is a self-limited and asymptomatic disease in 80% of infected adults [3], and recent scientific evidence have proposed a strong association between mothers infected with ZIKV and newborn's microcephaly [1b, 4]. Currently, the absence of well-established knowledge about the exact mechanisms by which Zika virus affects human brain development is stimulating researches worldwide.

A recent literature contribution has shown that human neural progenitor cells (hNPCs) infected with ZIKV present increased rates of cell death and deregulation of cell-cycle progression, in addition to transcriptional deregulation associated with apoptotic pathways [5]. Cell death, along with growth impairment and morphological abnormalities, was also detected by Brazilian researchers who studied Zika virus infection, Brazilian strain (ZIKV[BR]), in hNPCs [6]. Considering the evidence that ZIKV has tropism for brain cells, induces apoptosis, and modifies cell-cycle regulation, what would Zika virus infection cause in neural cancer cells?

The 17th most frequent cancer class worldwide are central nervous system tumors; out of those, glioblastomas (GBM) are the most common malignant brain tumor in adults, presenting extremely high morbidity and mortality [7]. After diagnosis, the prognosis for GBM is generally poor, with patients' presenting an average survival of about 11.5 months; additionally, the overall survival rate after treatment corresponds to less than 10% [8]. Classified as grade IV gliomas, GBMs typically present increased cellular proliferation rate, invasiveness, microvascular proliferation and necrosis, all significantly more intense when compared with more lenient gliomas [9]. The traditional treatment for GBM consists of maximal surgical resection, chemotherapy and radiotherapy. Neverthless, about 90% of surgical resections tumors recur, attesting the surgical incurability of GBMs and reduced life expectancy for these patients [10].

Taking into account the absence of an effective treatment for GBMs, and the ZIKV tropism for brain cells, along with its ability to induce neural cell death, the hypothesis formulated by the present contribution was that ZIKV would provoke cell death in glioblastomas through metabolic changes induced by the viral infection. Therefore, this study aimed at evaluating the metabolomic changes associated with ZIKV infection in glioblastoma cells. In addition, we proposed potential biochemical markers associated with cell death, so that future initiatives may benefit from these characteristics to interfere and improve the approaches for neural cancer treatment.

64 **EXPERIMENTAL SECTION**

65

66 *Cell culture and viral infection*

67 Human malignant M059J glioblastoma cells (ATCC Cat# CRL-2366, RRID:CVCL_0400), kindly

68 provided by Professor Marcelo Lancellotti, were seeded in cover slips of a 24-well plate, cultured in

69 RPMI medium and incubated at 37°C with 5% of CO_2. Upon 100% of confluence, ZIKV group was

70 submitted to viral transduction with $1x10^4$ PFU of Zika virus per well, while the control group (CT)

71 underwent the same conditions, except for viral inoculation. Eventually, the cell culture resulted in six

72 biological replicates for each condition studied. The viral strain obtained for this study corresponded

73 to the Brazilian ZIKV strain (BeH823339, GenBank KU729217) isolated from a patient in 2015, at the

74 State of Ceará, Brazil. Visual microscopic examinations were performed at 24 and 48 hours post-

75 infection, and bright field images were collected using a Zeiss Observer A1 microscope and processed

76 by Zeiss' AxioVision 4 software. Finally, both groups of cells were submitted to matrix laser

77 desorption/ionization mass spectrometry imaging (MALDI-MSI) analysis at each time point.

78

79 *Mass spectrometry analysis*

80 At each time point of infection, 24h and 48h, cell culture samples (6 cultures for each condition, all of

81 which analyzed in five different areas forming a total of 30 replicates for each condition) were

82 transferred to glass slides of 24x60 mm and covered with a 10 mg/mL solution of MALDI matrix α-

83 cyano-4-hydroxycinnamic acid (Sigma-Aldrich, St. Louis, MO) in 1:1 acetonitrile/methanol. Spectra

84 were acquired using a MALDI LTQ-XL (Thermo Scientific, San Jose, CA) at the mass range of 400 to

85 1400 *m/z*, in the negative ion mode, comprising five analytical replicates per group. Tandem mass

86 spectrometry data (MS/MS) was acquired in the same instrument, using Helium as the collision gas,

87 with energies for collision-induced dissociation ranging from 60–140 (arbitrary units). Spectra were

88 analyzed using XCalibur software (v. 2.4, Thermo Scientific, San Jose, CA).

89 *Structural elucidation*

90 Chemical markers were elucidated via the analysis of the MS/MS fragment profile of the ions that

91 were selected by statistical analysis. Structures were proposed with theoretical calculations modeling

92 for molecular fragmentation with the assistance of Mass Frontier software (v. 6.0, Thermo Scientific,

93 San Jose, CA). Structural confirmation of the cardiac glycoside Digoxin was carried out by

94 comparison with the MS/MS profile from the analytical standard of the compound.

95

96   *Semi quantification by MSI*

97   Chemical images of Digoxin were generated using the imaging feature of MALDI, and processed to
98   grayscale using the ImageQuest software (Thermo Scientific, San Jose, CA). Each respective replicate
99   from the control and ZIKV groups had their chemical image standardized and analyzed using ImageJ
100   software (National Institutes of Health, USA—open source). A non-dimensional value was assigned to
101   each image based on pixel intensity, so that the intensity/quantity ratio is established [11].

102   *Statistical analysis*

103   Partial least squares discriminant analysis (PLS-DA) was used as the method of choice to check for
104   association between groups. PLS-DA is a supervised method that uses multivariate regression
105   techniques to extract the characteristics that may evidence this association. The selection of lipids that
106   were characteristic for each group was carried out based on the impact that each feature had in the
107   model, i.e. the analysis of VIP (Variable Importance in Projection) scores. As a cutoff threshold, only
108   the chemical markers with a VIP score greater than 1.8 were analyzed. All analyses involving PLS-
109   DA and VIP scores were carried out using the online software MetaboAnalyst 3.0 [12]. The semi-
110   quantitative dataset was validated with a Student's *t*-test, and a *p*-value was significant if presented
111   values lower than 0.05. These data were processed using the software GraphPad Prism (v.3.0,
112   GraphPad Software, San Diego, CA).

113

114   **RESULTS**

115   *Cytopathic effects of ZIKV in GBM cells*

116   To identify ZIKV effects in neural cancer cells, human malignant M059J glioblastoma cells (GBM)
117   were infected with the Brazilian strain of Zika virus. Plates were analyzed at 24 and 48 hours after
118   infection. At the first time point evaluated (24 hours post-infection – hpi), GBM-ZIKV group (Fig. 1b)
119   presented slight cytopathic effects, as round and swollen cells, beyond syncytium formation, compared
120   to GBM-CT group (Fig. 1a), whilst 48hpi, GBM-ZIKV group (Fig. 1d) presented higher quantity of
121   round and swollen cells, syncytium formation and pronounced loss of cellular integrity compared with
122   the GBM-CT group (Fig. 1c), highlighting important cytopathic effects and cell death provoked by
123   viral infection at the second time point of analysis.
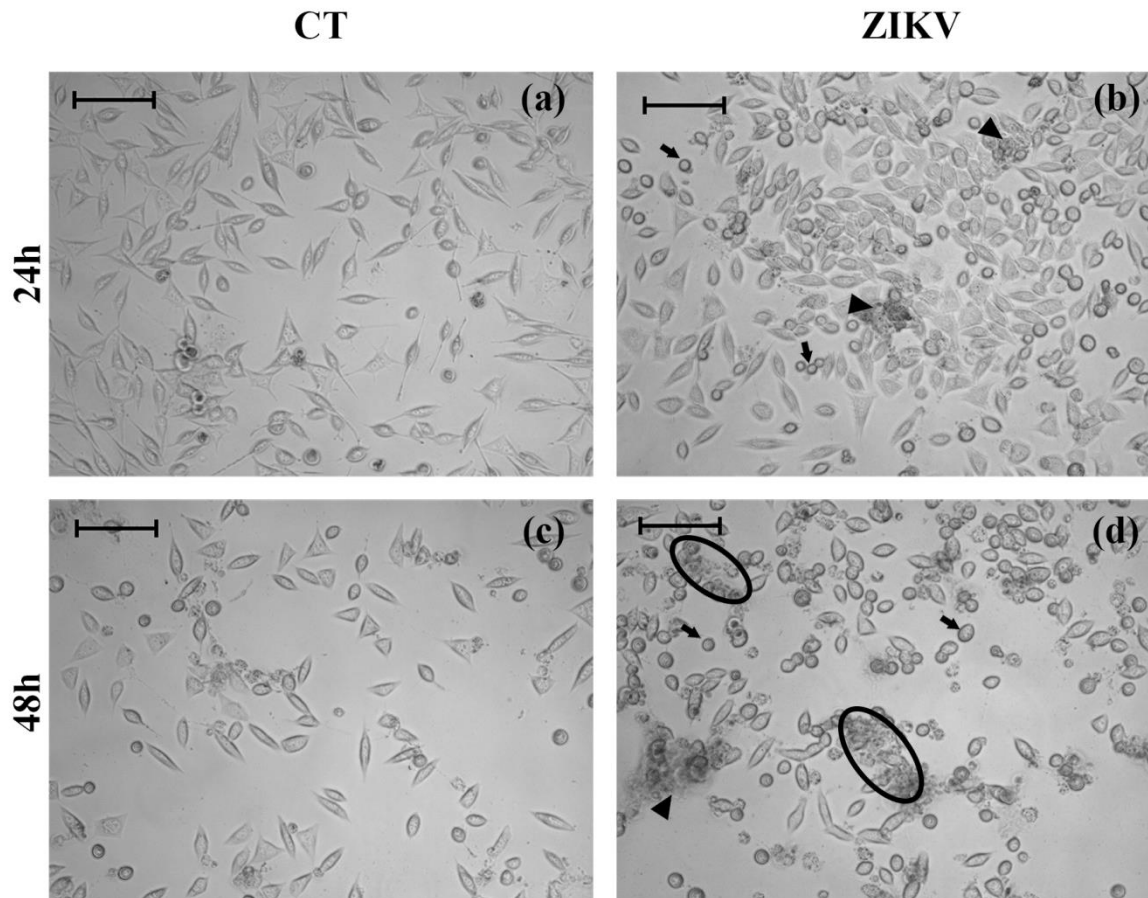
Figure 1. Zika virus induces cytopathic effects in glioblastoma cells

Optical photomicrographs of glioblastoma cells from both mock (GBM-CT) and Zika virus infected (GBM-ZIKV) groups, observed at 24 and 48 hours post-infection. (a) and (b) are microscopic images made at 24 hours post infection of GBM-CT and GBM-ZIKV samples, respectively; (c) and (d) are their counterparts, 48 hours post infection. Arrows indicate round and swollen cells; arrowheads points out to syncytium formation; ellipses demonstrate areas of cytopathic effect, where there is pronounced cellular loss of integrity. Scale bars 400x, 100 μm.

Metabolites identification through MALDI-MSI analysis

Metabolomics analytical approach was performed for both glioblastoma CT and ZIKV groups, aiming at analyzing the main metabolites involved with ZIKV infection. Mass spectrometric data (Fig. S1)

136   were submitted to a PLS-DA, which evidenced differences between metabolite composition in ZIKV-

137   infected cells versus uninfected control cells, for both time points of culture, as demonstrated in Fig. 2.

138   By establishing a threshold value of 1.8 for VIP scores, it was possible to elect biochemical markers

139   for Zika-virus infected group. For the group infected 24 hours post-infection, cells showed an

140   interesting cardiac glycoside (CG) - Digoxin (Table 1).

141
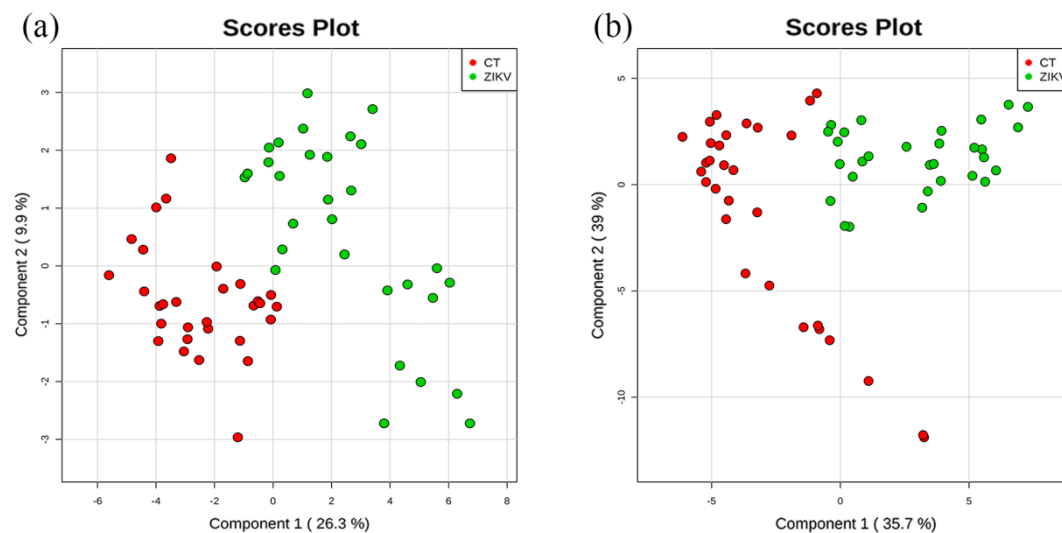
142   **Figure 2. Statistical evidences of metabolomics differences between mock and Zika virus-**

143   **infected groups.**

144   Scores plot derived from PLS-DA statistical analysis between mock (CT) and Zika virus-infected

145   (ZIKV) groups; (a) 24 hours post-infection (hpi), (b) 48 hpi. Each spot corresponds to one replicate

146   analyzed in the cell culture, where the red spots correspond to Mock infected group and the green

147   ones, to the ZIKV group. Each replicate corresponded to a distinct area of 10^3x10^3 µm in size in the

148   cell cultures.

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155 **Table 1.** Lipid chemical markers elected by PLS-DA VIP scores and elucidated by MS/MS for

156 glioblastoma cells infected with Zika virus (negative ion mode). The ion column represents the

157 theoretical mass identified for each biomarker.

Time of culture	Group	Class	Molecule	Precursor Ion (m/z)	Product Ions (MS/MS)	Molecule ID[a,b]
24 hpi	ZIKV	Cardiac glycoside	[Digoxin - H]⁻	779.4	735, 761, 691, 752	LMST01120023

158 Abbreviations: hpi, hours post-infection; ZIKV, Zika virus infected group.

159 a. LM: lipid maps ID.

160

161 *Digoxin semi-quantification*

162 Intending to corroborate the evidence that Digoxin was statistically important for the GBM-ZIKV

163 group, the chemical image for the ion $m/z = 779$ (Fig. 3a) was semi-quantified. The statistical analysis

164 is represented in Fig. 3b, providing enough evidence that Digoxin was significantly more expressed in

165 Zika virus infected groups, compared to GBM-CT groups.

166
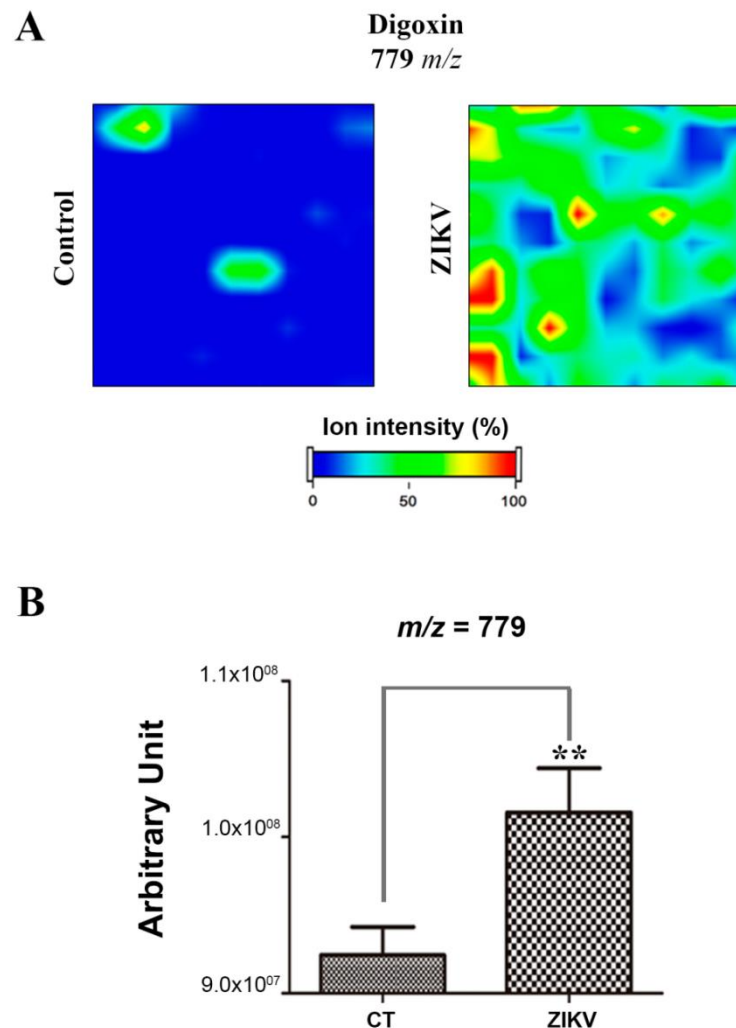
Figure 3. Digoxin is statistically more evident in Zika virus-infected group, 24 hpi.

(a)Digoxin ($m/z = 779$) ion image acquired from mock (control-CT) and Zika virus-infected (ZIKV) groups; (b) Semi-quantitative analysis of Digoxin observed in CT and ZIKV groups, with p = 0,002 (**).Differences on bar graphs were expressed by pixel intensities in the image for each group, as given by Oliveira *et al*., (2013).. Data presented as mean ± SEM, n = 30, Student's t-test, **p < 0.01.

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DISCUSSION

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175   Current research points out to metabolic changes during ZIKV infection which permitted to observe an

176   endogenous molecule that may bring light to alternative in aggressive cancer management. One of the

177   most interesting results obtained here is that malignant glioblastoma cells are indeed susceptible to

178   ZIKV infection, and show cytopathic effects such as altered shape, syncytium formation and loss of

179   cellular integrity in the culture plate, all of which denote cell death, over relatively short infection

180   times. Our findings are in line with a previous contribution that used microscopic analysis of ZIKV-

181   infected hNPs, in which cytopathic effects were evident as early as day 2, and cell death was

182   correlated with round and swollen shaped cells, and caspase-3 activation [13]. Similar results were

183   further shown for hNPCs infected with ZIKV, in which cell death was observed in association with

184   caspase-3 activation, involved in the regulation of apoptotic pathway [5]. This combined evidence, along

185   with the current findings in our study, corroborate to the hypothesis that ZIKV triggers the death of

186   neural cancer cells.

187   Despite the extensive research available to date, cancer management still poses a huge challenge for

188   healthcare professionals worldwide. Therefore, several members of the biomedical community have

189   been engaged in better understanding the biochemical basis and the physiology of cancer, intending to

190   improve the performance of cancer treatment. For this purpose, several studies are emerging and

191   bringing light to novel and unusual data about new alternatives for cancer treatment. Within this

192   scope, a class of molecules has been closely monitored based in clinical and scientific evidences:

193   cardiac glycosides (CGs) [14].

194   The most interesting and intriguing molecule identified in GBM-ZIKV group at the early stage of

195   infection (24 hours post-infection) belongs to cardiac glycosides group, more specifically the

196   cardenolides subgroup [15], and consists of Digoxin. Cardiac glycosides are sterol lipids, well known as

197   secondary metabolites in plants, however, there are few studies that describe these molecules as

198   endogenous compounds in mammals [16]. By the end of the past century, scientific studies, including

199   mass spectrometric analyses showed that cardiac glycosides could be identified in human tissues and

200   fluids, such as adrenal glands, kidneys, brain, plasma and urine [17]. Endogenous *de novo* biosynthesis

201   of CGs was firstly reported by Qazzaz and colleagues (2004), attesting the previous evidences cited

202   above [17a, 18]. Furthermore, previous studies have demonstrated that patients with breast cancer, under

203   cardiac glycosides treatment present reduced recurrence and mortality rates compared with the

204   untreated group [19]. *In vitro* studies have shown that innumerous cancer cell lines, like breast,

205   melanoma and neuroblastoma cells present reduced multiplication rate and increased cell death after

9

206   treatment with CGs [20]. In addition, cardiac glycosides like Digoxin were also evaluated regarding

207   GBM cells response, and the treatment induced proliferation impairment and apoptotic phenotype [21].

208   Considering the existence of several *in vivo* and *in vitro* data showing that CGs induces cancer cell

209   death, it is plausible to admit that ZIKV infection brought on endogenous synthesis of Digoxin in

210   glioblastoma cells. This contrasts directly with the group with mock-infected cells, where the presence

211   of this cardiac glycoside was not observed. Thus, we propose that this phenomenon is probably one of

212   the triggers for neuronal cell death induced by Zika virus.

213   Digoxin is a well-known potent inhibitor of the Na^+-K^+-ATPase ion pump, which is responsible for the

214   establishment and maintenance of the electrochemical gradient across plasma membrane [22]. Taking

215   into account that neuronal signaling depends on generation and maintenance of membrane potential,

216   Digoxin may disrupt ion homeostasis and disturb neuronal excitability, both for healthy and cancer

217   neuronal cells. Furthermore, the sodium-potassium pump inhibitory effect leads to intracellular

218   increase in sodium and calcium levels, which mediates several signaling pathways, including

219   proliferation, differentiation and apoptosis [22b, 23]. Although Na^+-K^+-ATPase is an enzyme present in

220   every mammalian cell, in this case, where synthesis of digoxin is induced by Zika virus infection, the

221   harmful effects of cardenolides would be restricted to infected cells, what would protect the non-

222   infected ones.

223   Beyond the effects cited above, some studies have shown possible mechanisms of action for cardiac

224   gangliosides in cancer cells, suggesting they might interfere in different signaling pathways, what

225   initiates with their interaction with Na^+-K^+-ATPase ion pump. This first step triggers changes at

226   different signaling cascades via distinct proteins, as Src (Proto-oncogene tyrosine kinase),

227   Phosphoinositide 3-kinase (PI3K), Phospolipase C and Ras pathways, which together might result in

228   tumor cell death through apoptosis or autophagy mechanisms [24]. Some studies have also shown that

229   digoxin induces apoptosis via activation of Caspase-3, which ends with generation of reactive oxygen

230   species (ROS) and cell death [25]. All these signaling cascades together will affect DNA translation and

231   consequently alter synthesis of proteins which are involved with growth, survive and cell cycle control

232   [26]. Digoxin, for instance, showed reduction of hypoxia-inducible factor 1 (HIF-1α) gene expression

233   and protein synthesis at cancer cells, which impairs the role of HIF-1α as an important transcription

234   factor for cancer angiogenesis. Although digoxin inhibition of HIF-1α occurs, HIF-1α expression is

235   not affected when cells are treated with a vector containing only coding sequences, generating mRNA

236   without untranslated regions (UTRs). That means that digoxin might function as an mRNA

237   translational regulator at UTRs from HIF-1α mRNA generated [27]. In case of cancers, negative

238   interference with vascular support for GBM cells is of great value for cancer management, and digoxin

239   induced by Zika virus might be an alternative to weaken tumor cells and reduce glioblastoma growth.

240   Although the exact mechanism of action of cardiac glycosides still needs to be elucidated, Digoxin

241   seems to be a robust candidate for neural cancer management.

242   Although Digoxin was not elected as a significant biomarker for GBM-ZIKV group in the later stage

243   of infection (48 hours post-infection), it indicates that Digoxin was important at the first hours after

244   ZIKV infection. It is likely Digoxin, in the first 24 hours of infection, was synthesized and induced

245   intracellular changes that triggered cell death pathways after 48 hours. The reduction of living cells

246   after 48 hpi impairs digoxin election as a statistical biomarker. Furthermore, the biochemical changes

247   caused by Digoxin might have overlapped its own expression and reduced its significance at statistical

248   analysis.

249   Therefore, the present study has shown that malignant glioblastoma cells were susceptible to ZIKV

250   infection, which was verified through cytopathic effects over infection time. Therefore, microscopic

251   observations combined with the metabolomics data revealed in the present study suggest that Digoxin,

252   synthesized after stimulation by Zika virus, may be responsible for glioblastoma cells death. Based on

253   these results, remains the question whether a genetically engineered Zika virus might be an alternative

254   for cancer treatment.

255   Since Zika virus presents tropism for neural progenitor cells, originated from either fetal or adult

256   brains [13, 28], the consequences of the infection in adults are not as severe as in fetuses [28], as fetuses

257   present a higher number of neural stem cells compared to adults [29]. This vulnerability of neural

258   precursors to Zika virus has recently been confirmed by Chavali *et al*., 2017. This study demonstrated

259   that cells with higher expression of protein Musashi-1 (MSI-1), like glioblastoma and neuronal

260   precursor cells, presented high viral replication rates. The presence of MSI-1 was essential for Zika

261   virus replication, once MSI-1 was responsible for promoting viral RNA translation in stem cells [30].

262   Thus, researchers concluded that Zika virus might present tropism for immature neuronal cells because

263   MSI-1 is highly expressed on it, but absent at totally differentiated neuronal cells, what explains the

264   deleterious effects of ZIKV infection at fetuses' brains, differently of adult's brains. Considering that

265   glioblastomas might be originated from cancerous neural stem cells (immature cells) [31], it is plausible

266   to consider them as important Zika virus targets.

267   Therefore, through the sensitivity of glioblastoma cells to Zika virus infection demonstrated in the

268   present study, and the viral selectivity for progenitor cells, we propose that the synthesis of Digoxin

269   induced by Zika virus might be an innovative alternative for neural cancer management. In a practical

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270   example, members from the Food and Drug Administration – FDA and European Medicines Agency

271   (EMA) have already approved the use of genetically engineered virus, called Talimogene

272   laherparepvec (T-VEC) to treat advanced melanoma [32]. Clinical trials with different oncolytic viruses

273   are already in course, as in the case of avian adenovirus, myxoma virus, feline panleukopenia virus,

274   polio virus, among others [33]. An interesting case was observed during a clinical trial with an

275   engineered measles virus, where a myeloma patient entered on complete remission after the treatment

276   [34]. One of the reasons for these trials is that many viruses preferentially infect cancer cells, and if so,

277   healthy cells could be safe while viral infection eliminates the tumor [32b]. Therefore, the use of

278   oncolytic viruses is a promising strategy, and further studies must be taken forward to verify the link

279   between synthesis of Digoxin and Zika virus infection. ZIKV may be, therefore, ultimately genetically

280   engineered and be a strong alternative to glioblastoma management, in an effort to provide better

281   quality of life and survival rates for these patients.

282   In addition to the possibility of using ZIKV for neural cancer management, an alternative to explore

283   the results obtained from this research is to evaluate the potential use of Digoxin for Zika virus

284   diagnosis. Current ZIKV diagnostic tools are based on the detection of antibodies (immunoassays)

285   and/or viral components, whether viral proteins or RNA. The routine diagnostic method for ZIKV

286   infection is RT-PCR, which might be complemented with MAC-ELISA assay [35]. However, the low

287   levels of viremia after one week of the illness onset and the cross-reactivity of flavivirus antibodies

288   makes difficult to obtain the correct diagnosis [36]. Taking into account the need for highest specificity

289   and sensitivity for ZIKV diagnosis, Digoxin might be a potential biomarker for that. Further studies

290   may be conducted to verify the presence of this chemical marker in biological samples from infected

291   patients, in addition to evaluate the specificity of cardiac glycosides for ZIKV infection. Once Digoxin

292   is validated as a ZIKV biomarker, it is possible to ultimately apply the metabolomics strategy for the

293   diagnosis of Zika virus infection.

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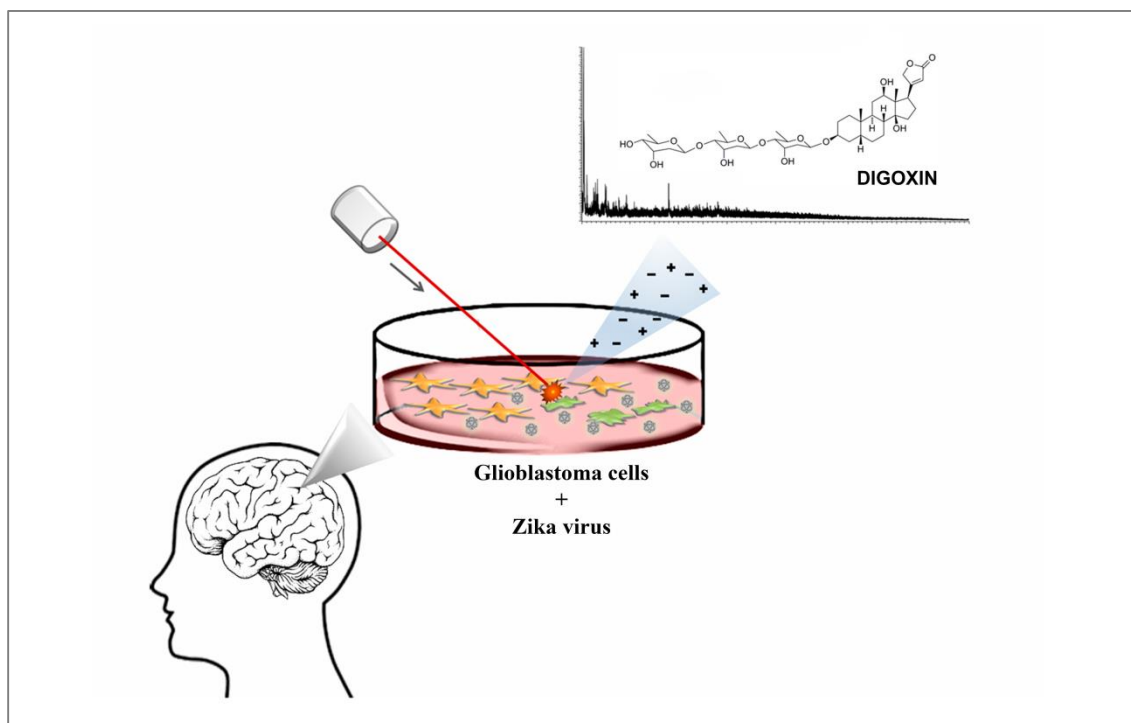
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