

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

3 **Authors:** Estela de O. Lima¹, Tatiane M. Guerreiro¹, Carlos F. O. R. Melo¹, Diogo N. de Oliveira¹,
4 Daisy Machado², Marcelo Lancelloti², Rodrigo R. Catharino¹

5 **Affiliations:** ¹Innovare Biomarkers Laboratory, School of Pharmaceutical Sciences, University of
6 Campinas - UNICAMP, Campinas, SP, 13083-877, Brazil

7 ²Laboratory of Biotechnology, School of Pharmaceutical Sciences, University of Campinas –
8 UNICAMP, Campinas, SP, 13083-859, Brazil

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.

30 INTRODUCTION

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

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

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

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

63

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 CO₂. Upon 100% of confluence, ZIKV group was
70 submitted to viral transduction with 1x10⁴ 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*

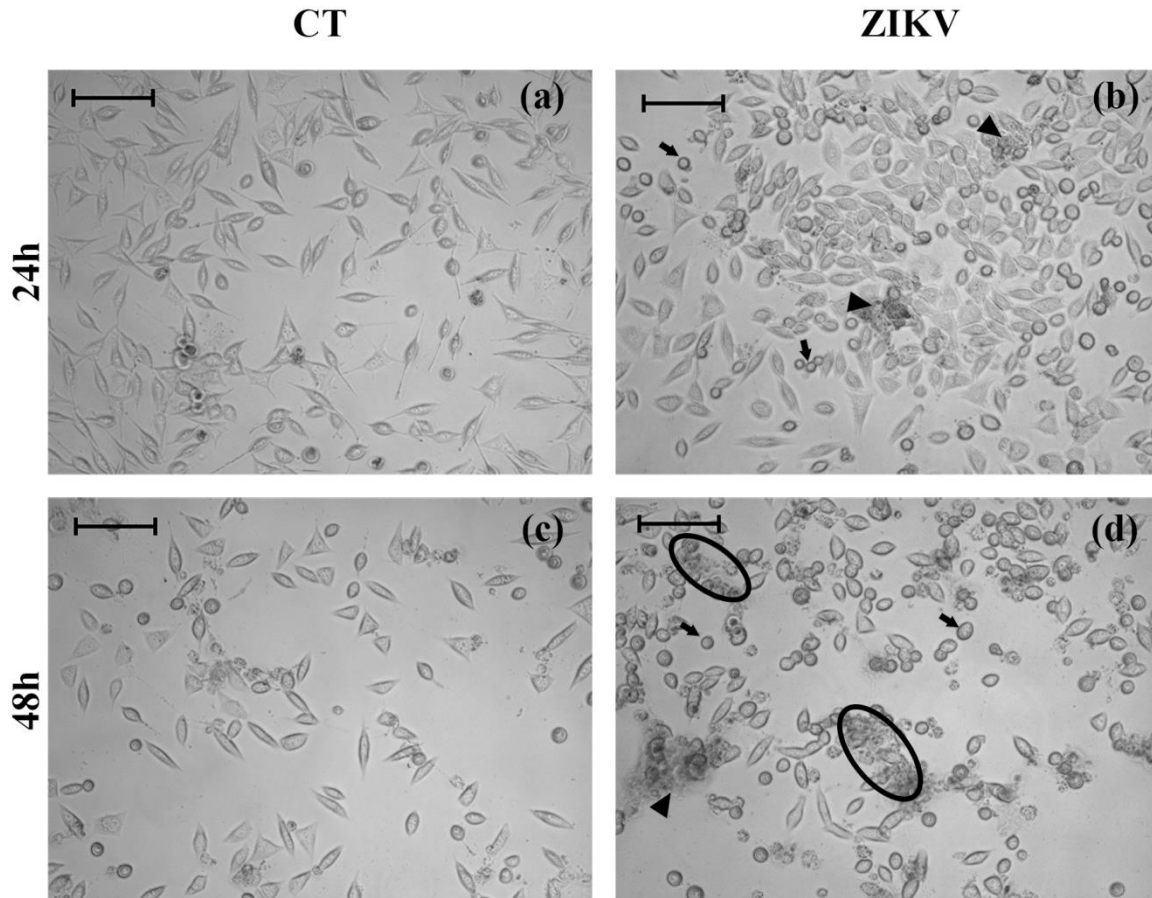
103 Partial least squares discriminant analysis (PLS-DA) was used as the method of choice to check for
104 association between groups. PLS-DA is a supervised method that uses multivariate regression
105 techniques to extract the characteristics that may evidence this association. The selection of lipids 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.



124

125 **Figure 1. Zika virus induces cytopathic effects in glioblastoma cells**

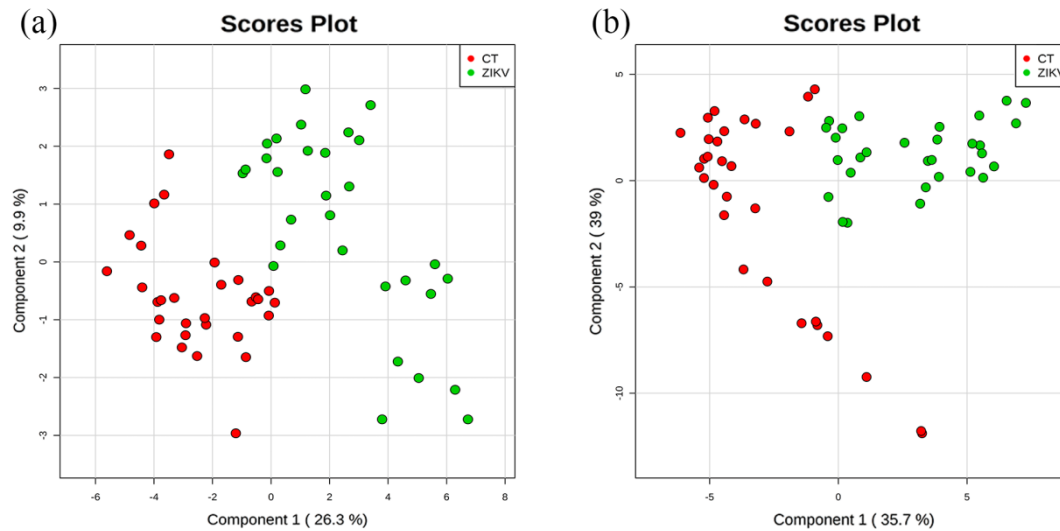
126 Optical photomicrographs of glioblastoma cells from both mock (GBM-CT) and Zika virus infected
127 (GBM-ZIKV) groups, observed at 24 and 48 hours post-infection. (a) and (b) are microscopic images
128 made at 24 hours post infection of GBM-CT and GBM-ZIKV samples, respectively; (c) and (d) are
129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

132

133 *Metabolites identification through MALDI-MSI analysis*

134 Metabolomics analytical approach was performed for both glioblastoma CT and ZIKV groups, aiming
135 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 $10^3 \times 10^3 \mu\text{m}$ in size in the
148 cell cultures.

149

150

151

152

153

154

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

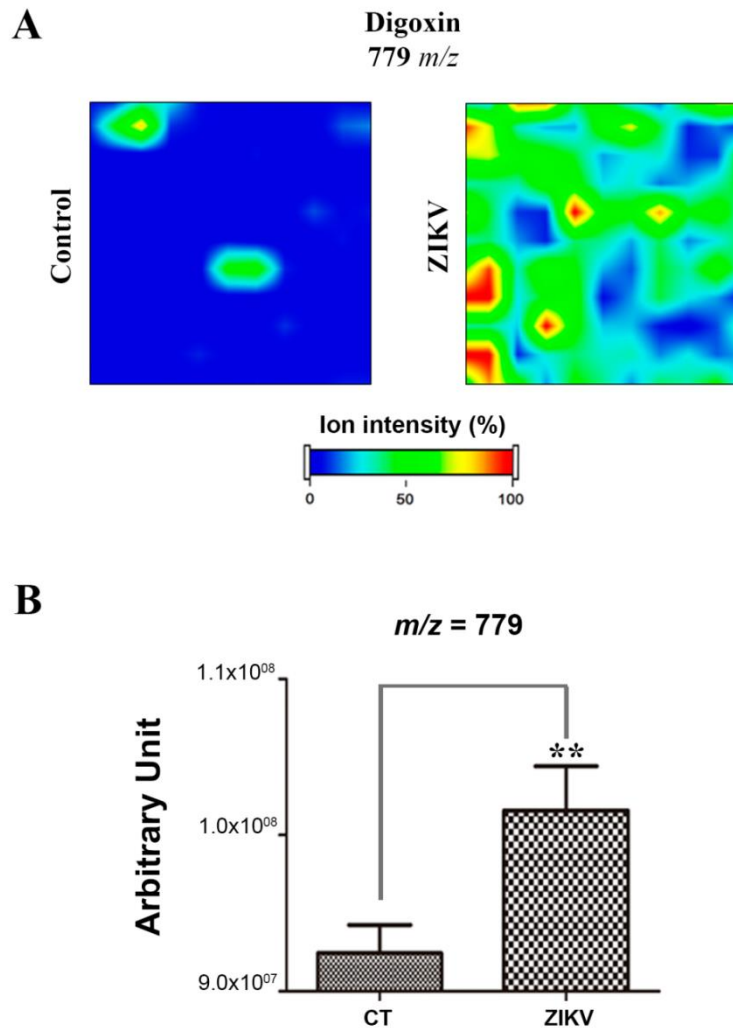
158 Abbreviations: hpi, hours post-infection; ZIKV, Zika virus infected group.

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

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

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

172

173

174 DISCUSSION

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 activation¹³. 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 pathway⁵. 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 subgroup¹⁵, 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 mammals¹⁶. 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 urine¹⁷. 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 group¹⁹. *In vitro* studies have shown that innumerable cancer cell lines, like breast,
205 melanoma and neuroblastoma cells present reduced multiplication rate and increased cell death after

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), Phospholipase 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-1 α) gene expression
233 and protein synthesis at cancer cells, which impairs the role of HIF-1 α as an important transcription
234 factor for cancer angiogenesis. Although digoxin inhibition of HIF-1 α occurs, HIF-1 α 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-1 α 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 fetuses²⁸, as fetuses
257 present a higher number of neural stem cells compared to adults²⁹. 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 cells³⁰.
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

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 melanoma³². 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 others³³. 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
276³⁴. 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 assay³⁵. 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 diagnosis³⁶. 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.

294 **ACKNOWLEDGMENTS**

295 Innovare Laboratory would like to thank Professor José Butori Lopes de Faria and Diego Andreazzi
296 Duarte for providing the infrastructure of the microscopy lab of the *Laboratório de Microdiabetes*
297 *from the Clínica Médica (FCM)*, essential for cell imaging. We also acknowledge São Paulo Research
298 Foundation (FAPESP, Process Nos. 11/50400-0 and 15/06809-1 for RRC, 14/00302-0 for CZE. We
299 also thank Coordination for the Improvement of Higher Level Personnel (CAPES) for the fellowships
300 from CFORM (PROEX: 1645986), EOL (PNPD: 1578388) and TMG(PROEX: 1489740). DNO
301 acknowledges the Plano Nacional de Enfrentamento ao *Aedes aegypti* e à Microcefalia [*Brazilian Plan*

302 for Fighting *Aedes aegypti* and *Microcephaly*] for the fellowship under process No.
303 88887.137889/2017-00. All researchers involved declare no conflicts of interest of any nature.

304 AUTHOR INFORMATION NOTES

305 Corresponding author: e-mail: rrcatharino@gmail.com

306 Phone: 55 19 35219138

307 REFERENCES

- 308 1. (a) Campos, G. S.; Bandeira, A. C.; Sardi, S. I., Zika virus outbreak, Bahia, Brazil. *Emerging*
309 *infectious diseases* **2015**, *21* (10), 1885; (b) Saúde, M. d., Confirmação do Zika virus no Brasil. 2015;
310 (c) Fauci, A. S.; Morens, D. M., Zika virus in the Americas—yet another arbovirus threat. *New*
311 *England Journal of Medicine* **2016**, *374* (7), 601-604; (d) Organization, P. A. O. W. H., Zika
312 suspected and confirmed cases reported by countries and territories in the Americas Cumulative cases,
313 2015-2016. Updated as of 6 October 2016. .
314 [https://www.google.com.br/url?sa=t&rct=j&q=&esrc=s&source=web&cd=3&cad=rja&uact=8&ved=](https://www.google.com.br/url?sa=t&rct=j&q=&esrc=s&source=web&cd=3&cad=rja&uact=8&ved=0ahUKEwiE28-5wdjPAhXGkZAKHekKCWMQFgguMAI&url=http%3A%2F%2Fwww.paho.org%2Fhq%2Findex.php%3Foption%3Dcom_docman%26task%3Ddoc_view%26Itemid%3D270%26gid%3D36428%26lang%3Den&usg=AFQjCNG3ysgpiKKmCV3NH1KKHQRMB07qGQ&sig2=A0xNPhD71TbCuVDjo-IzTQ&bvm=by.135475266.d.Y2I)
315 [0ahUKEwiE28-](https://www.google.com.br/url?sa=t&rct=j&q=&esrc=s&source=web&cd=3&cad=rja&uact=8&ved=0ahUKEwiE28-5wdjPAhXGkZAKHekKCWMQFgguMAI&url=http%3A%2F%2Fwww.paho.org%2Fhq%2Findex.php%3Foption%3Dcom_docman%26task%3Ddoc_view%26Itemid%3D270%26gid%3D36428%26lang%3Den&usg=AFQjCNG3ysgpiKKmCV3NH1KKHQRMB07qGQ&sig2=A0xNPhD71TbCuVDjo-IzTQ&bvm=by.135475266.d.Y2I)
316 [5wdjPAhXGkZAKHekKCWMQFgguMAI&url=http%3A%2F%2Fwww.paho.org%2Fhq%2Findex.p](https://www.google.com.br/url?sa=t&rct=j&q=&esrc=s&source=web&cd=3&cad=rja&uact=8&ved=0ahUKEwiE28-5wdjPAhXGkZAKHekKCWMQFgguMAI&url=http%3A%2F%2Fwww.paho.org%2Fhq%2Findex.php%3Foption%3Dcom_docman%26task%3Ddoc_view%26Itemid%3D270%26gid%3D36428%26lang%3Den&usg=AFQjCNG3ysgpiKKmCV3NH1KKHQRMB07qGQ&sig2=A0xNPhD71TbCuVDjo-IzTQ&bvm=by.135475266.d.Y2I)
317 [hp%3Foption%3Dcom_docman%26task%3Ddoc_view%26Itemid%3D270%26gid%3D36428%26lan](https://www.google.com.br/url?sa=t&rct=j&q=&esrc=s&source=web&cd=3&cad=rja&uact=8&ved=0ahUKEwiE28-5wdjPAhXGkZAKHekKCWMQFgguMAI&url=http%3A%2F%2Fwww.paho.org%2Fhq%2Findex.php%3Foption%3Dcom_docman%26task%3Ddoc_view%26Itemid%3D270%26gid%3D36428%26lang%3Den&usg=AFQjCNG3ysgpiKKmCV3NH1KKHQRMB07qGQ&sig2=A0xNPhD71TbCuVDjo-IzTQ&bvm=by.135475266.d.Y2I)
318 [g%3Den&usg=AFQjCNG3ysgpiKKmCV3NH1KKHQRMB07qGQ&sig2=A0xNPhD71TbCuVDjo-](https://www.google.com.br/url?sa=t&rct=j&q=&esrc=s&source=web&cd=3&cad=rja&uact=8&ved=0ahUKEwiE28-5wdjPAhXGkZAKHekKCWMQFgguMAI&url=http%3A%2F%2Fwww.paho.org%2Fhq%2Findex.php%3Foption%3Dcom_docman%26task%3Ddoc_view%26Itemid%3D270%26gid%3D36428%26lang%3Den&usg=AFQjCNG3ysgpiKKmCV3NH1KKHQRMB07qGQ&sig2=A0xNPhD71TbCuVDjo-IzTQ&bvm=by.135475266.d.Y2I)
319 [IzTQ&bvm=by.135475266.d.Y2I](https://www.google.com.br/url?sa=t&rct=j&q=&esrc=s&source=web&cd=3&cad=rja&uact=8&ved=0ahUKEwiE28-5wdjPAhXGkZAKHekKCWMQFgguMAI&url=http%3A%2F%2Fwww.paho.org%2Fhq%2Findex.php%3Foption%3Dcom_docman%26task%3Ddoc_view%26Itemid%3D270%26gid%3D36428%26lang%3Den&usg=AFQjCNG3ysgpiKKmCV3NH1KKHQRMB07qGQ&sig2=A0xNPhD71TbCuVDjo-IzTQ&bvm=by.135475266.d.Y2I), 2016.
- 320 2. Brunner, M.; Koskinen, A. M., Biology and Chemistry of Sphingosine-Related Metabolites.
321 *Current Organic Chemistry* **2004**, *8* (17), 1629-1645.
- 322 3. Lopes, N.; Nozawa, C.; Linhares, R. E. C., Características gerais e epidemiologia dos
323 arbovírus emergentes no Brasil. *Revista Pan-Amazônica de Saúde* **2014**, *5* (3), 55-64.
- 324 4. Mlakar, J.; Korva, M.; Tul, N.; Popović, M.; Poljšak-Prijatelj, M.; Mraz, J.; Kolenc, M.;
325 Resman Rus, K.; Vesnaver Vipotnik, T.; Fabjan Vodusek, V., Zika virus associated with
326 microcephaly. *New England Journal of Medicine* **2016**, *374* (10), 951-958.
- 327 5. Tang, H.; Hammack, C.; Ogden, S. C.; Wen, Z.; Qian, X.; Li, Y.; Yao, B.; Shin, J.; Zhang, F.;
328 Lee, E. M., Zika virus infects human cortical neural progenitors and attenuates their growth. *Cell stem*
329 *cell* **2016**, *18* (5), 587-590.
- 330 6. Cugola, F. R.; Fernandes, I. R.; Russo, F. B.; Freitas, B. C.; Dias, J. L.; Guimarães, K. P.;
331 Benazzato, C.; Almeida, N.; Pignatari, G. C.; Romero, S., The Brazilian Zika virus strain causes birth
332 defects in experimental models. *Nature* **2016**.

- 333 7. (a) Wen, P. Y.; Kesari, S., Malignant gliomas in adults. *New England Journal of Medicine*
334 **2008**, 359 (5), 492-507; (b) Stewart, B.; Wild, C. P., World cancer report 2014. *World* **2016**.
- 335 8. Lutterbach, J.; Guttenberger, R.; Pagenstecher, A., Gliosarcoma: a clinical study.
336 *Radiotherapy and Oncology* **2001**, 61 (1), 57-64.
- 337 9. Kleihues, P.; Sobin, L. H., World Health Organization classification of tumors. *Cancer* **2000**,
338 88 (12), 2887-2887.
- 339 10. Berens, M. E.; Giese, A., "... those left behind." Biology and Oncology of Invasive Glioma
340 Cells. *Neoplasia* **1999**, 1 (3), 208-219.
- 341 11. De Oliveira, D. N.; de Bona Sartor, S.; Ferreira, M. S.; Catharino, R. R., Cosmetic analysis
342 using matrix-assisted laser desorption/ionization mass spectrometry imaging (MALDI-MSI).
343 *Materials* **2013**, 6 (3), 1000-1010.
- 344 12. Xia, J.; Sinelnikov, I. V.; Han, B.; Wishart, D. S., MetaboAnalyst 3.0—making metabolomics
345 more meaningful. *Nucleic acids research* **2015**, 43 (W1), W251-W257.
- 346 13. Hanners, N. W.; Eitson, J. L.; Usui, N.; Richardson, R. B.; Wexler, E. M.; Konopka, G.;
347 Schoggins, J. W., Western Zika Virus in Human Fetal Neural Progenitors Persists Long Term with
348 Partial Cytopathic and Limited Immunogenic Effects. *Cell reports* **2016**.
- 349 14. Prassas, I.; Diamandis, E. P., Novel therapeutic applications of cardiac glycosides. *Nature*
350 *reviews Drug discovery* **2008**, 7 (11), 926-935.
- 351 15. Gokhale, M. S., *Pharmacognosy*. Pragati Books Pvt. Ltd.: 2008.
- 352 16. Melero, C. P.; Medarde, M.; San Feliciano, A., A short review on cardiotonic steroids and
353 their aminoguanidine analogues. *Molecules* **2000**, 5 (1), 51-81.
- 354 17. (a) Shaikh, I.; Lau, B.; Siegfried, B.; Valdes, R., Isolation of digoxin-like immunoreactive
355 factors from mammalian adrenal cortex. *Journal of Biological Chemistry* **1991**, 266 (21), 13672-
356 13678; (b) Mathews, W. R.; DuCharme, D. W.; Hamlyn, J. M.; Harris, D. W.; Mandel, F.; Clark, M.
357 A.; Ludens, J. H., Mass spectral characterization of an endogenous digitalislike factor from human
358 plasma. *Hypertension* **1991**, 17 (6 Pt 2), 930-935; (c) Bagrov, A. Y.; Fedorova, O. V.; Dmitrieva, R. I.;
359 Howald, W. N.; Hunter, A. P.; Kuznetsova, E. A.; Shpen, V. M., Characterization of a urinary
360 bufodienolide Na⁺, K⁺-ATPase inhibitor in patients after acute myocardial infarction. *Hypertension*
361 **1998**, 31 (5), 1097-1103.
- 362 18. Qazzaz, H.; Jortani, S.; Poole, J.; Valdes, R., Evidence for presence of a reduced form of
363 digoxin-like immunoreactive factor (dihydro-DLIF) in mammalian tissues. *Clinical chemistry* **1996**,
364 42 (7), 1092-1099.
- 365 19. (a) Stenkvist, B.; Bengtsson, E.; Eklund, G.; Eriksson, O.; Holmquist, J.; Nordin, B.;
366 Westman-Naeser, S., Evidence of a modifying influence of heart glucosides on the development of

- 367 breast cancer. *Analytical and quantitative cytology* **1979**, 2 (1), 49-54; (b) Stenkvist, B.; Bengtsson, E.;
368 Dahlqvist, B.; Eriksson, O.; Jarkrans, T.; Nordin, B., Cardiac glycosides and breast cancer, revisited.
369 *The New England journal of medicine* **1982**, 306 (8), 484; (c) Stenkvist, B., Is digitalis a therapy for
370 breast carcinoma? *Oncology reports* **1999**, 6, 493-496.
- 371 20. (a) López-Lázaro, M.; Pastor, N.; Azrak, S. S.; Ayuso, M. J.; Austin, C. A.; Cortés, F.,
372 Digitoxin inhibits the growth of cancer cell lines at concentrations commonly found in cardiac
373 patients. *Journal of Natural Products* **2005**, 68 (11), 1642-1645; (b) Newman, R. A.; Yang, P.;
374 Hittelman, W. N.; Lu, T.; Ho, D. H.; Ni, D.; Chan, D.; Vijjeswarapu, M.; Cartwright, C.; Dixon, S.,
375 Oleandrin-mediated oxidative stress in human melanoma cells. *J Exp Ther Oncol* **2006**, 5 (3), 167-
376 181; (c) Kulikov, A.; Eva, A.; Kirch, U.; Boldyrev, A.; Scheiner-Bobis, G., Ouabain activates
377 signaling pathways associated with cell death in human neuroblastoma. *Biochimica et Biophysica Acta*
378 *(BBA)-Biomembranes* **2007**, 1768 (7), 1691-1702.
- 379 21. Joshi, A. D.; Parsons, D. W.; Velculescu, V. E.; Riggins, G. J., Sodium ion channel mutations
380 in glioblastoma patients correlate with shorter survival. *Molecular cancer* **2011**, 10 (1), 17.
- 381 22. (a) Schatzmann, H.; Räss, B., Inhibition of the active Na-K-transport and Na-K-activated
382 membrane ATP-ase of erythrocyte stroma by ouabain. *Helvetica physiologica et pharmacologica acta*
383 **1965**, 65 (1); (b) Kaplan, J. H., Biochemistry of na, K-ATPase. *Annual review of biochemistry* **2002**,
384 71 (1), 511-535.
- 385 23. Dolmetsch, R. E.; Xu, K.; Lewis, R. S., Calcium oscillations increase the efficiency and
386 specificity of gene expression. *Nature* **1998**, 392 (6679), 933-6.
- 387 24. (a) Liang, M.; Cai, T.; Tian, J.; Qu, W.; Xie, Z.-J., Functional characterization of Src-
388 interacting Na/K-ATPase using RNA interference assay. *Journal of biological chemistry* **2006**, 281
389 (28), 19709-19719; (b) Schoner, W.; Scheiner-Bobis, G., Endogenous and exogenous cardiac
390 glycosides: their roles in hypertension, salt metabolism, and cell growth. *American Journal of*
391 *Physiology-Cell Physiology* **2007**, 293 (2), C509-C536; (c) Xie, Z.; Cai, T., Na⁺-K⁺-ATPase-
392 mediated signal transduction: from protein interaction to cellular function. *Molecular interventions*
393 **2003**, 3 (3), 157; (d) Liu, J.; Tian, J.; Haas, M.; Shapiro, J. I.; Askari, A.; Xie, Z., Ouabain interaction
394 with cardiac Na⁺/K⁺-ATPase initiates signal cascades independent of changes in intracellular Na⁺ and
395 Ca²⁺ concentrations. *Journal of Biological Chemistry* **2000**, 275 (36), 27838-27844.
- 396 25. (a) Akimova, O. A.; Lopina, O. D.; Hamet, P.; Orlov, S. N., Search for intermediates of Na⁺,
397 K⁺-ATPase-mediated [Na⁺]_i/[K⁺]_i-independent death signaling triggered by cardiotonic steroids.
398 *Pathophysiology* **2005**, 12 (2), 125-135; (b) Huang, Y.-T.; Chueh, S.-C.; Teng, C.-M.; Guh, J.-H.,
399 Investigation of ouabain-induced anticancer effect in human androgen-independent prostate cancer
400 PC-3 cells. *Biochemical pharmacology* **2004**, 67 (4), 727-733.

- 401 26. (a) Kometiani, P.; Liu, L.; Askari, A., Digitalis-induced signaling by Na⁺/K⁺-ATPase in
402 human breast cancer cells. *Molecular pharmacology* **2005**, *67* (3), 929-936; (b) Haas, M.; Wang, H.;
403 Tian, J.; Xie, Z., Src-mediated inter-receptor cross-talk between the Na⁺/K⁺-ATPase and the
404 epidermal growth factor receptor relays the signal from ouabain to mitogen-activated protein kinases.
405 *Journal of Biological Chemistry* **2002**, *277* (21), 18694-18702.
- 406 27. (a) Zhang, H.; Qian, D. Z.; Tan, Y. S.; Lee, K.; Gao, P.; Ren, Y. R.; Rey, S.; Hammers, H.;
407 Chang, D.; Pili, R., Digoxin and other cardiac glycosides inhibit HIF-1 α synthesis and block tumor
408 growth. *Proceedings of the National Academy of Sciences* **2008**, *105* (50), 19579-19586; (b) Manalo,
409 D. J.; Rowan, A.; Lavoie, T.; Natarajan, L.; Kelly, B. D.; Shui, Q. Y.; Garcia, J. G.; Semenza, G. L.,
410 Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. *Blood* **2005**, *105*
411 (2), 659-669.
- 412 28. Li, H.; Saucedo-Cuevas, L.; Regla-Nava, J. A.; Chai, G.; Sheets, N.; Tang, W.; Terskikh, A.
413 V.; Shresta, S.; Gleeson, J. G., Zika virus infects neural progenitors in the adult mouse brain and alters
414 proliferation. *Cell Stem Cell* **2016**, *19* (5), 593-598.
- 415 29. (a) Eriksson, P. S.; Perfilieva, E.; Björk-Eriksson, T.; Alborn, A.-M.; Nordborg, C.; Peterson,
416 D. A.; Gage, F. H., Neurogenesis in the adult human hippocampus. *Nature medicine* **1998**, *4* (11),
417 1313-1317; (b) Nunes, M. C.; Roy, N. S.; Keyoung, H. M.; Goodman, R. R.; McKhann, G.; Jiang, L.;
418 Kang, J.; Nedergaard, M.; Goldman, S. A., Identification and isolation of multipotential neural
419 progenitor cells from the subcortical white matter of the adult human brain. *Nature medicine* **2003**, *9*
420 (4), 439-447.
- 421 30. Chavali, P. L.; Stojic, L.; Meredith, L. W.; Joseph, N.; Nahorski, M. S.; Sanford, T. J.;
422 Sweeney, T. R.; Krishna, B. A.; Hosmillo, M.; Firth, A. E.; Bayliss, R.; Marcelis, C. L.; Lindsay, S.;
423 Goodfellow, I.; Woods, C. G.; Gergely, F., Neurodevelopmental protein Musashi 1 interacts with the
424 Zika genome and promotes viral replication. *Science* **2017**.
- 425 31. (a) Galli, R.; Binda, E.; Orfanelli, U.; Cipelletti, B.; Gritti, A.; De Vitis, S.; Fiocco, R.; Foroni,
426 C.; Dimeco, F.; Vescovi, A., Isolation and characterization of tumorigenic, stem-like neural precursors
427 from human glioblastoma. *Cancer research* **2004**, *64* (19), 7011-7021; (b) Singh, S. K.; Hawkins, C.;
428 Clarke, I. D.; Squire, J. A.; Bayani, J.; Hide, T.; Henkelman, R. M.; Cusimano, M. D.; Dirks, P. B.,
429 Identification of human brain tumour initiating cells. *nature* **2004**, *432* (7015), 396-401.
- 430 32. (a) Food, U.; Administration, D., FDA approves first-of-its-kind product for the treatment of
431 melanoma. Press release. October 27, 2015. 2015; (b) Ledford, H., Cancer-fighting viruses near
432 market. *Nature* **2015**, *526* (7575), 622-623.
- 433 33. Vähä-Koskela, M. J.; Heikkilä, J. E.; Hinkkanen, A. E., Oncolytic viruses in cancer therapy.
434 *Cancer letters* **2007**, *254* (2), 178-216.

- 435 34. Russell, S. J.; Federspiel, M. J.; Peng, K.-W.; Tong, C.; Dingli, D.; Morice, W. G.; Lowe, V.;
436 O'connor, M. K.; Kyle, R. A.; Leung, N. In *Remission of disseminated cancer after systemic oncolytic*
437 *virotherapy*, Mayo Clinic Proceedings, Elsevier: 2014; pp 926-933.
- 438 35. Petersen, L. R.; Jamieson, D. J.; Powers, A. M.; Honein, M. A., Zika virus. *New England*
439 *Journal of Medicine* **2016**, *374* (16), 1552-1563.
- 440 36. (a) Bearcroft, W., Zika virus infection experimentally induced in a human volunteer.
441 *Transactions of the Royal Society of Tropical Medicine and Hygiene* **1956**, *50* (5), 442-448; (b)
442 Lanciotti, R. S.; Kosoy, O. L.; Laven, J. J.; Velez, J. O.; Lambert, A. J.; Johnson, A. J.; Stanfield, S.
443 M.; Duffy, M. R., Genetic and serologic properties of Zika virus associated with an epidemic, Yap
444 State, Micronesia, 2007. *Emerging infectious diseases* **2008**, *14* (8), 1232.

445

446

447

448

449

450

451

452

453

454

455

456

457

458

459

460

461

462

463

464

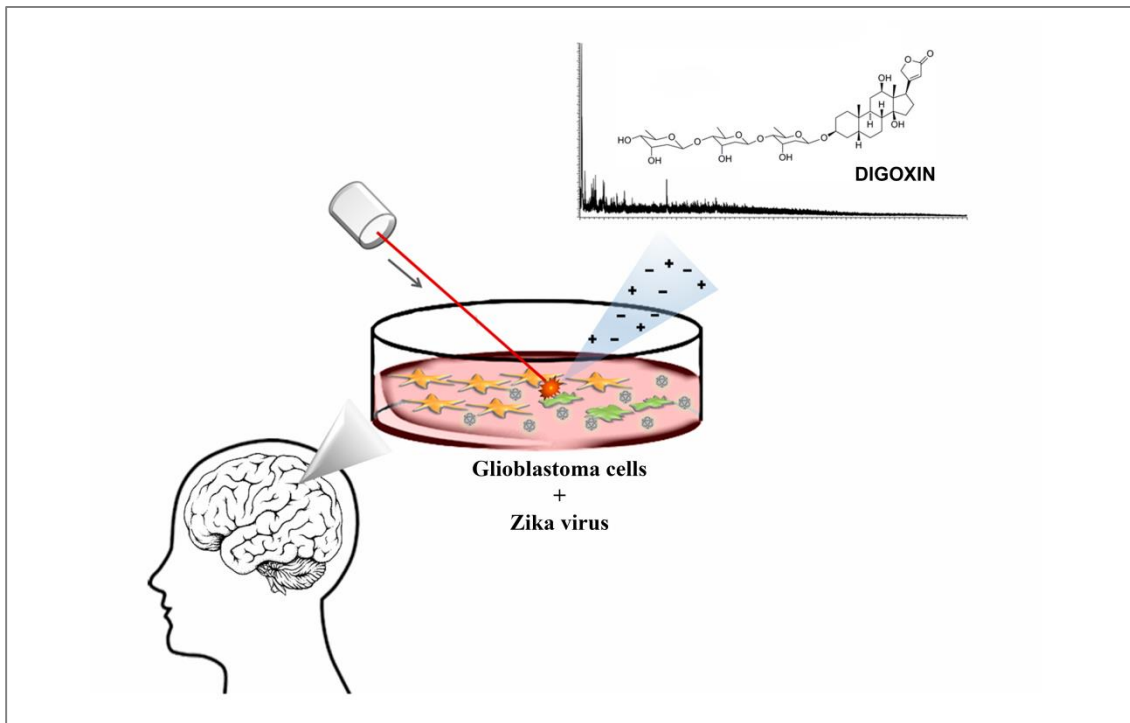
465

466

467

468

469 **TABLE OF CONTENTS:**



470