Role of Sonic hedgehog signaling in cell cycle, oxidative stress, and autophagy of temozolomide resistant glioblastoma

Jessica R. Honorato1,2 | Rachel A. Hauser-Davis3 | Enrico M. Saggioro4 | Fábio V. Correia5 | Sidney F. Sales-Junior6 | Lorena O. S. Soares5 | Leandro da R. Lima5 | Vivaldo Moura-Neto1,2 | Giselle P. de F. Lopes2,7 | Tania C. L. de S. Spohr1,2

1Laboratório de Biomedicina do Cérebro, Instituto Estadual do Cérebro Paulo Moreira (IECPN), Secretaria de Estado de Saúde, Rio de Janeiro, Brazil
2Programa de Pós-Graduação em Anatômia Patológica, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
3Instituto de Anatomia e Citologia, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil
4Departamento de Ciências da Saúde, Universidade Federal do Rio de Janeiro (UNIRIO), Rio de Janeiro, Brazil
5Departamento de Ciências Naturais, Universidade Federal do Rio de Janeiro (UNIRIO), Rio de Janeiro, Brazil
6Department of Biotecnologia Marinha, Instituto de Estudos do Mar Almirante Paulo Moreira (IEAPM)/Coordenação de Pesquisa, Instituto Nacional de Câncer (INCA), Rio de Janeiro, Brazil

Correspondence
Giselle P. Lopes, Departamento de Biotecnologia Marinha, Instituto de Estudos do Mar Almirante Paulo Moreira (IEAPM), Rua Kioto 253, Praia dos Anjos, Araraquara, SP, Brazil. Email: giselle.lopes@inca.gov.br

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Abstract
The first-line chemotherapy treatment for Glioblastoma (GBM) - the most aggressive and frequent brain tumor - is temozolomide (TMZ). The Sonic hedgehog (SHH) pathway is involved with GBM tumorigenesis and TMZ chemoresistance. The role of SHH pathway inhibition in the potentiation of TMZ's effects using T98G, U251, and GBM11 cell lines is investigated herein. The combination of GANT-61 and TMZ over 72 hr suggested a synergistic effect. All TMZ-resistant cell lines displayed a significant decrease in cell viability, increased DNA fragmentation and loss of membrane integrity. For T98G cells, G2/M arrest was observed, while U251 cells presented a significant increase in reactive oxygen species production and catalase activity. All the cell lines presented acidic vesicles formation correlated to Beclin-1 overexpression. The combined treatment also enhanced GLI1 expression, indicating the presence of select resistant cells. The selective inhibition of the SHH pathway potentiated the cytotoxic effect of TMZ, thus becoming a promising in vitro strategy for GBM treatment.

KEYWORDS
GANT-61, glioblastoma, Sonic hedgehog, temozolomide

Abbreviations: AO, Acridine orange; ATCC, American Type Culture Collection; AVOs, Acidic vesicles organelles; BBB, Blood-brain barrier; BSA, Bovine serum albumin; CAT, Catalase; CDI, Coefficient of drug interaction; CSCs, Cancer stem cells; DAPI, 6-Diamino-2-phenylindole; DCF, Dichlorofluorescein; DCFH-DA, Dichloro-dihydro-fluorescein diacetate; DMEM/F12, Dulbecco’s medium supplemented with F-12; DMSO, Dimethyl-sulfoxide; DTNB, 5,5-Dithiobis-2-nitrobenzoic acid; EDTA, Ethylenediamine tetra acetic acid; EMT, Epithelial–mesenchymal transition; FBS, Fetal bovine serum; G1, Primary growth phase; G2, Secondary growth phase; GANT-61, Gli antagonist 61; GBM, Glioblastoma; M, Cell division phase; MDR1, Multi-drug resistance gene; MGMT, O-6-methylguanine-DNA methyltransferase enzyme; MITC, Monomethyl triazeno imidazole carboxamide; MTT, 3-[4,5-Dimethylthiazol-2-(5)-diphenyl tetrazolium bromide; OD, Optical density; PBS, Phosphate buffered saline; PFA, Paraformaldehyde; PI, Propidium iodide; ROS, Reactive oxygen species; S, DNA synthesis phase; SHH, Sonic hedgehog; TGFβs, Transforming growth factor beta; TMZ, Temozolomide; WHO, World Health Organization; WNT, Wingless.
**INTRODUCTION**

Glioblastoma (GBM) is the most lethal form of primary brain cancer, which is responsible for several deaths each year (Shen et al., 2016). Due to its high aggressiveness, the World Health Organization (WHO) has ranked GBM as a Grade IV tumor (Louis et al., 2016). Currently, the first-line therapy for GBM treatment combines surgical resection, radiotherapy, and chemotherapy with temozolomide (TMZ; Lai et al., 2018). TMZ is an important alkylating agent that transposes the blood–brain barrier and is responsible for DNA damage when hydrolyzed to an active metabolite, monomethyl triazeno imidazolyl carboxamide (MTIC), resulting in tumors cell death (Agarwala & Kirkwood, 2000). However, even with this first-line therapy, patient prognosis is limited with a median survival of only 13.5 months (Li et al., 2016). GBM cells are resistant to TMZ due to repair mechanisms such as increased O-6-methylguanine-DNA methyltransferase (MGMT) enzyme expression and the presence of the multidrug resistance (MDR1) gene expression (Goellner et al., 2011; Munoz, Rodriguez-Cruz, Walker, Greco, & Rameshwar, 2015). Moreover, cancer stem cells (CSCs) in the tumor mass are also responsible for tumor recurrence, as these cells are chemoresistant (Lai et al., 2018). Until now, clinical trials have shown that combined therapy may extend a patient’s life by 2 months (Perry et al., 2017). Thus, the discovery of new associated pharmaco-therapies to overcome this tumor type is urgent.

The tumorigenesis process involves the transformation of a normal cell into a tumor cell, through the activation or inhibition of many signaling pathways (Ramdasi, 2016). It is often observed that signaling pathways vital to embryo development, such as transforming growth factor beta (TGFβs), wingless (WNT) and Sonic hedgehog (SHH) pathways are positively modulated during tumorigenesis (Geraldo et al., 2018; Golestaneh & Mishra, 2005; Roth et al., 2000). The SHH signaling cascade is crucial for neural tube patterning, a process that depends on stem cell proliferation to provide cell mass for neural development (Belgacem, Hamilton, Shim, Spencer, & Borodinsky, 2016; Uygur et al., 2016). When the SHH pathway is reactivated during GBM progression it displays the ability to maintain CSC profiles in the tumor mass, thereby interfering with patient treatment (Biswa et al., 2015). The positive modulation of the SHH pathway leads to the nuclear translocation of GLI (GLI1, GLI2, and GLI3) proteins, which are a family of transcription factors responsible for regulating stemness genes, such as OCT4, SOX2, and NANOG, involved in the maintenance of CSCs properties (Carballo, Honorato, De Lopes, & Spohr, 2018; Sasaki, Hui, Nakafuku, & Kondoh, 1997; You, Guo, & Huang, 2018). To overcome this issue, many SHH pathway-selective inhibitory drugs have been developed, such as GLI-antagonist 61 (GANT-61) and cyclopamine (Li et al., 2016).

GANT-61 is a hexahydropropirimicidine derivate that is first described during a cellular screening to discover new molecules that can selectively inactivate the SHH pathway (Lauth, Bergstrom, Shimokawa, & Toftgard, 2007). GANT-61 prevents the GLI1 transcription factor from binding with nuclear DNA by interacting with a groove between GLI1 zinc fingers 2 and 3, blocking the transcription activity (Agyeman, Jha, Mazumdar, & Houghton, 2014; Gonnissen et al., 2016). This compound has displayed effects on cancer cells, such as cytotoxicity associated with apoptosis, autophagy induction, prevention of DNA repair, and CSCs self-renewal arrest (Agyeman et al., 2014; Gonnissen et al., 2016). The potential of GANT-61 in silencing the SHH pathway, thereby reverting the CSC phenotype makes this compound a possible ally in the search for new GBM treatment drugs.

Since combination therapy is described as a cornerstone for cancer treatment, the hypothesis formulated for this study is that the combination of GANT-61 and TMZ may improve the therapeutic strategy for GBM. To test this hypothesis in vitro, different GBM cell lines incubated with TMZ and GANT-61 were individually or concomitantly used, to evaluate the molecular mechanisms involved in cytotoxicity, cell cycle, oxidative stress, differentiation, and autophagy in this type of tumor.

**MATERIAL AND METHODS**

### 2.1 Reagents

All the culture reagents as well as secondary antibodies, conjugated to Alexa Fluor 488 and 546, were obtained from Life Technologies (Carlsbad, CA). Dulbecco’s medium supplemented with F-12 (DMEM/F-12) and fetal bovine serum (FBS) were purchased from Gibco (MA). 4–6-Diamino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), TMZ, dimethyl-sulfoxide (DMSO), bovine serum albumin (BSA), propidium iodide (PI), citrate buffer, acridine orange (AO), dichloro-dihydro-fluorescein diacetate (DCFH–DA), Ellman’s reagent (DTNB, 5,5-dithiobis-2-nitrobenzoic acid), 1-chloro-2,4-dinitrobenzene, and glutathione (GSH) were purchased from Sigma-Aldrich (St. Louis, MO). H2O2 was purchased from Merck (Darmstadt, Germany). Triton X-100 and ethylenediamine tetra acetic acid (EDTA) were purchased from LGC Biotechnology (São Paulo, Brazil). Paraformaldehyde (PFA) was purchased from Janssen (Brazil). Ki67 and anti-Beclin1 antibody were obtained from Dako (Glostrup, Denmark). The anti-GLI1 antibody was obtained from Millipore (Billerica, MA). The anti-Beclin1 antibody was obtained from BD Biosciences (CA).

### 2.2 Human GBM cell culture

The human GBM cell lines T98G and U251 were obtained from the American Type Culture Collection (ATCC) and GBM11 was obtained in collaboration with Dr. Jorge Marcondes (Clementino Fraga Filho University Hospital, Rio de Janeiro, Brazil) according to the regulations set forth by the Federal University of Rio de Janeiro/Research Ethics Committee (DAHEICB 015) and the rules of the Brazilian Ministry of Health Ethics Committee (CONEP 2340). The GBM11 cell line was characterized from the biopsy of a 57-year-old male patient with recurrent GBM previously treated...
with TMZ and radiotherapy (Bačka-Silva et al., 2017). The three different cell lines were cultured in DMEM/F-12% and 10% FBS at 37°C in a humidified atmosphere with 5% CO₂. The ATCC cell lines were authenticated by their STR profile and all the cell lines were free from mycoplasma infection.

### 2.3 | Biochemical assay

For the biochemical assays, the GBM cell lines were seeded at the following cell densities: 50,000/cm² for T98G, 21,875/cm² for U251 and 87,500/cm² for GBM11, into 96-well plates for 24, 48, and 72 hr with the vehicle 0.8% DMSO (CONTROL), GANT-61 (20 μmol/L), TMZ (400 μmol/L) or TMZ (400 μmol/L), and GANT-61 (20 μmol/L) concomitantly at 5% of SFB.

#### 2.3.1 | MTT assay

The MTT (5 mg/ml) was added at the final concentration of 10% over 2 hr to the previously prepared GBM cell lines. Subsequently, purple formazan crystals were dissolved in it by adding 100 μl DMSO/well for 20 min and absorbances were determined according to optical density (OD) at 570 nm (Victor Multilabel Plate Reader, PerkinElmer). The experiments were conducted at least three times, and five replicates per experimental condition. The results were analyzed considering the relative proportion to the OD control median and converted to a percentage of cellular viability. The coefficient of drug interaction (CDI) was calculated using the equation CDI = AB/(A × B), where AB is the relative cell viability of the combination; A or B is the relative cell viability of the single agent; CDI < 1 implies a synergistic effect, CDI = 1 implies an additive effect and CDI > 1 refers to an antagonistic effect (Li et al., 2016).

#### 2.3.2 | Immunofluorescence

This assay was performed as previously described (Spohr, Dezonne, Rehen, & Gomes, 2011). After the treatment, the GBM cells were fixed in 4% PFA (20 min) followed by permeabilization with 0.1% Triton ×100 (5 min) and the blocking of nonspecific binding with BSA 5% (1 hr) at room temperature. Next, the cells were incubated with primary antibodies, such as mouse anti-Ki67 (1:200), mouse anti-Vimentin (1:100), rabbit anti-Gli1 (1:500), and mouse anti-Beclin1 (1:100). After the primary antibody incubation at 4°C overnight, the cells were washed with phosphate buffered saline (PBS) and incubated with monoclonal or polyclonal secondary antibodies conjugated to Alexa Fluor 488 (1:750) or Alexa Fluor 546 (1:1,000) at 37°C for 1 hr and 20 min. Subsequently, the cells were washed again with PBS and the nuclei were stained with DAPI (1 μg/ml; 5 min) at room temperature. The slides were observed under fluorescence microscopy (DMI8 Leica Microsystems). The fluorescence intensity was quantified by the integrated density median using the ImageJ software (NIH). The experiments were conducted at least three times with ten replicates per experimental condition. The results were analyzed considering the relative proportion to the control fluorescence median.

#### 2.3.3 | Flow cytometry

**Cell cycle and DNA fragmentation**

The DNA content representative of the cell cycle phases and fragmentation was accessed through PI incorporation. After the GBM cell treatment, the cells were washed with PBS, detached with trypsin (0.25%)/EDTA (0.02%) and centrifuged (Microcentrifuge, Eppendorf 5430R) at 27 g (5 min). Then, the cell cycle solution containing PI 50 mg/ml, Triton 0.3%, and citrate buffer 4 mmol/L was added to the cell suspension for 15 min at room temperature. Flow cytometry was performed using a BD FACS Calibur (BD Biosciences) on the FL3 channel. The cell cycle was assessed through the primary growth phase (G0/G1), DNA synthesis phase (S), and secondary growth phase/cell division phase (G2/M) phases, and DNA fragmentation was evaluated using the sub-G0 phase. The experiments were conducted at least three times, and a minimum of 10,000 events were acquired. The analysis was performed using the Summit 4.3 software. The results were analyzed considering the percentage of cells in each cell cycle phase per experimental condition.

**Membrane integrity by propidium iodide**

The membrane integrity representative of early cell death was detected through PI incorporation. After the GBM cell treatment, the cells were washed with PBS, detached with trypsin (0.25%)/EDTA (0.02%) and centrifuged (Microcentrifuge, Eppendorf 5430R) at 27 g (5 min). Next, PBS with PI 50 μg/ml was added at the moment of cytometry acquisition. Flow cytometry was performed using a BD FACS Calibur and the PI fluorescence was measured on FL3 channel. Membrane integrity was assessed through the percentage of FL3 positive cells. The experiments were conducted at least three times and a minimum of 10,000 events were acquired. The analysis was performed using the Summit 4.3 software. The results were analyzed considering the percentage of PI positive cells per experimental condition.

**Acidic vesicles organelles**

The AO fluorochrome was used to evaluate the presence of acidic vesicles organelles (AVOs), which are indicative of autophagy. After the GBM cell treatment and centrifugation were performed as described above PBS was added to the negative control and AO (1 μmol/L) was added to each sample. Flow cytometry was performed using a BD FACS Calibur and AO fluorescence was measured on the FL1 and FL3 channel. The AVOs were assessed through the percentage of FL1 and FL3 positive cells. The experiments were conducted at least three times and a minimum of 10,000 events were acquired. The analysis was performed using the Summit 4.3 software. The results were analyzed considering the proportion relative to the control fluorescence median of AO.
Reactive oxygen species

The occurrence of reactive oxygen species (ROS) was determined using the non-polar dye DCFH-DA that emits fluorescence when oxidized with dichlorofluorescein (DCF; Kalyanaramana et al., 2013). After the GBM cell treatment and centrifugation were performed as described above, PBS was added to the negative control and DCFH-DA (40 μmol/L) was added to each samples. PI was added at the moment of the events acquisition to distinguish and excluded dead cells from the analysis. Flow cytometry was performed using a BD FACSCalibur, DCF fluorescence was measured on FL1 channel and PI fluorescence was measured by FL3 channel. The ROS were assessed through the percentage of FL1 positive cells. The experiments were conducted at least three times and a minimum of 10,000 events were acquired. The analysis was performed using the Summit 4.3 software. The results were analyzed considering the proportion relative to the control fluorescence median of DCF.

2.3.4 Antioxidants detection

After the GBM cell treatment, the cells were washed with cold PBS and scraped off at 4°C. More cold PBS was added, and the suspension was stored at −80°C until quantification.

Nonenzymatic biomarker – glutathione

Glutathione (GSH) extraction was carried out according to Beutler (1963) with modifications recommended by (Wilhelm Filho, Torres, Zaniboni-Filho, & Pedrosa, 2005). The samples were first homogenized in PBS 0.1 mol/L, pH 6.5, containing 0.25 mol/L sucrose and 1 mmol/L EDTA and then centrifuged for 30 min at 11,000 g. The partially purified supernatants were then treated with Ellman’s reagent (DTNB, 5,5-dithiobis-2-nitrobenzoic acid) followed by being incubated for 15 min in the dark; absorbances were determined at 412 nm on a V‐530 UV‐vis spectrophotometer (Jasco, São Paulo, Brazil). GSH concentrations were estimated using GSH as an external standard. The R² analytical curves were above 0.995 and, thus, considered adequate for the GSH analyses. The experiments were conducted at least three times with three replicates per experimental condition.

Enzymatic biomarkers – catalase activity

Catalase (CAT) activity was kinetically determined according to (Aebi, 1984) in a reaction medium containing H₂O₂, whose depletion was constantly monitored for 15 s on the same V-530 UV-vis spectrophotometer cited previously at 240 nm. To normalize the CAT concentrations, the total protein contents, were determined at 240 nm. To normalize the CAT and total protein content analyses. The experiments were conducted at least three times with three replicates per experimental condition.

2.4 Statistical analyses

A Shapiro–Wilk normality test was conducted and the results were expressed as median ± min-max or mean ± standard deviation (SD) distribution. The one-way analysis of variance (ANOVA) and Tukey’s tests were used to compare the means or medians of each experimental condition. The significance threshold of p was considered < .05. The statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA).

3 RESULTS

3.1 GANT-61 potentiaed the TMZ effect by reducing the cell viability

TMZ associated with surgical resection and radiation therapy is the standard treatment for patients suffering from GBM. However, approximately 55% of these patients are resistant to TMZ (Karachi, Dastmalchi, Mitchell, & Rahman, 2018). Since the aberrant activation of the SHH pathway has been reported to be associated with GBM resistance (Munoz et al., 2015), the in vitro response of the three different GBM cell lines to TMZ and to GANT-61 were assessed. To this end, the three different human GBM cell lines T98G, U251, and GBM11 were incubated with increasing concentrations of TMZ (100, 200, and 400 μmol/L) or GANT-61 (5,10, and 20 μmol/L) for 24, 48, and 72 hr, respectively. Then, cell viability was accessed by the MTT assay. Figure 1 indicates that the cell lines presented a different response to TMZ treatment. SHH inhibition by GANT-61 did not appear to influence the viability of the cell lines as determined by the MTT assay. Thus, TMZ at a concentration of 400 μmol/L was applied to all further experiments, as its significantly decreased cell viability after 48 (U251: 77.8 ± 0.8%; T98G: 80.0 ± 1.27%; U251: 68.9 ± 3.4%) of treatment. Since no GANT-61 concentrations affected the cell viability after 24, 48, or 72 hr of incubation, 20 μmol/L was chosen.

As mentioned previously, it is believed that GBM’s resistance to TMZ is mainly due to the high expression of MGMT enzyme, which reverses the TMZ effect on the DNA (Sarkaria et al., 2008). In addition, it is known that GBM’s aggressiveness is closely related to the presence of CSCs in the tumor mass and that the SHH pathway is also crucial for the maintenance and proliferation of CSCs (Boyle & Kochetkova, 2014; Kahler, Mooney, Natsumeda, Steiger, & Maciàczyk, 2017). In this context, the selective inhibition of the SHH pathway with GANT-61 in the cell lines was evaluated, to assess the potentiation of the cytotoxic effect of TMZ. To this end, the T98G, U251, and GBM11 cell lines were incubated with GANT-61 20 μmol/L, TMZ 400 μmol/L or TMZ 400 μmol/L and GANT-61 20 μmol/L concomitantly for 48 and 72 hr (Figure 2a).

The TMZ+GANT-61 treatment significantly decreased the cell viability of all the cell lines after 48 (T98G: 67.0 ± 7.2%; U251: 74.0 ± 2.4; GBM11: 85.7 ± 1.8%) and 72 hr (T98G: 50.3 ± 19.4%; U251: 55.4 ± 5.0; GBM11: 71.8 ± 19.9%) of treatment (Figure 2a).
T98G and U251 were more sensitive to the combined treatment than GBM11, against the recurrent tumor. Moreover, 48 hr of treatment with TMZ+GANT‐61 significantly reduced the cell viability of GBM11 (85.7 ± 1.8%) compared with treatment with only TMZ. To confirm the MTT assays results, the cell lines were plated on coverslips and incubated under the same conditions described above for 72 hr. The viable cells stained with DAPI were counted after the treatment and we found a similar reduction in TMZ+GANT‐61 condition as compared to the MTT assays at 72 hr (T98G: 41.9 ± 6.3%; U251: 58.2 ± 16.4%; GBM11: 71.0 ± 35.4%; Figure 2b). Moreover, we observed a synergistic effect between TMZ and GANT‐61 in all the three different human GBM cell lines (CDI < 1) for 48 hr and 72 hr (Table 1).

3.2 Association of GANT‐61 and TMZ induced the selection of resistant cells

The transcription factor GLI1 is one of the main SHH pathway effectors, where a high expression during tumorigenesis leads to the CSCs phenotype being considered the main cause of tumor progression and therapeutic resistance (Lo, Zhu, Cao, Aldrich, & Ali-Osman, 2009; Wu & Schöler, 2014). Moreover, SHH signaling through the GLI1→CSCs axis is described as per its role in GBM resistance to TMZ since CSCs preferentially activate the DNA damage checkpoint response and display an increased ability for DNA repair (Bao et al., 2006; Chen et al., 2014). We evaluated whether SHH inhibition with GANT‐61 is capable of sensitizing cells to TMZ, examining the GLI1...
and vimentin expression (Figure 3). To this end, the T98G, U251 and GBM11 cell lines were incubated with GANT-61 20 μmol/L, and TMZ 400 μmol/L or TMZ 400 μmol/L + GANT-61 20 μmol/L for 72 hr. A significantly increased expression of nuclear GLI1 when the T98G (481.0 ± 334.0 – 547.0%) and U251 (4,208.0 ± 1,032.0 – 5,505.0%) cell lines were incubated with TMZ + GANT-61 was observed, suggesting that these cells were the resistant ones. GBM11 did not present significant differences in GLI1 expression.

GBM also displays increased potential to migrate and infiltrate brain tissue, the epithelial–mesenchymal transition (EMT) phenomenon
that TMZ was able to induce DNA damage in the T98G cell line. TMZ+GANT phase under the same treatment conditions (TMZ: 20.6 ± 19.6 μmol/L or TMZ 400 μmol/L for 72 hr.

A significant increase in vimentin expression was observed in U251 (209.3 ± 150.0–237.4%) and GBM11 (213.9 ± 179.5–214.1%) after the combined incubation with TMZ and GANT-61. Interestingly TMZ alone seemed to increase the vimentin expression in T98G, albeit not statistically significantly (Figure 3).

3.3 | TMZ alone or combined with GANT-61 induced cell cycle arrest

TMZ is a DNA alkylating agent and its cytotoxicity effect is mediated by the addition of methyl groups at guanines (N7 and O6 sites) and adenines (O3 site) in genomic DNA (Lee, 2016), thereby inducing cell cycle arrest in the G2/M phase in the GBM cell lines (Wang, Chang, Lin, Wei, & Shin, 2017). On the other hand, the reactivation of the SHH signaling cascade is able to induce cellular proliferation through opposing signals for physiologic growth arrest (Chen, Sims-Setti, Osti, & Pelicci, 2013). Among EMT-related proteins over-expressed in GBM, vimentin is a major cytoskeletal proteins associated with cellular structure, and has been associated with tumor progression and poor patient prognosis (Liu et al., 2018; Liu, Lin, Tang, & Wang, 2015; Satelli & Li, 2011). Since the SHH pathway activation has been pointed as one of the EMT-promoting agents (Islam et al., 2016), the expression of vimentin in the T98G, U251, and GBM11 cell lines was accessed, in addition evaluating the effects of SHH inhibition in combination with TMZ on vimentin expression. The T98G, U251, and GBM11 cell lines were incubated with GANT-61 20 μmol/L, and TMZ 400 μmol/L or TMZ 400 μmol/L + GANT61 20 μmol/L for 72 hr.

Regarding the autophagy mechanism, all the GBM cell lines treated with GANT-61 concomitantly with TMZ presented increased AVOs production (T98G: 296.9 ± 148.7–409.0%; U251: 261.5 ± 214.5–285.1%; GBM11: 213.4 ± 165.9–221.2%) or TMZ (T98G: 187.8 ± 124.1–295.5%; U251: 237.9 ± 138.4–246.7%; GBM11: 198.5 ± 191.5–221.2%) alone (Figure 5a). The results suggest an increase in catalasem activity only in the U251 cells after the synergistic treatment. However, no significant changes in the antioxidant enzyme profile in the cell lines were observed (Figure 5b).

3.4 | Cellular oxidative mechanisms respond to the combined treatment by inducing autophagy

The cellular oxidative system plays a crucial role in the regulation of signaling pathways that are responsible for cell death and survival in cancer (Seyfried, Marschall, & Fulda, 2016). It is known that TMZ and GANT-61 can activate the oxidative system by ROS production in tumor cells (Lim et al., 2015; Seyfried et al., 2016). In recent years, oxidative stress has also been linked to autophagy induction through ROS production - by acting as the main intracellular signal that sustains the autophagy process - and enhanced autophagy is able to induce tumor cell death (Filomeni, De Zio, & Cecconi, 2015; Wang et al., 2018).

To evaluate whether GANT-61 associated with TMZ has the potential to alter the cellular oxidative mechanisms and induce autophagy we treated the cells as describe above. All the cell lines presented significantly increased ROS production when incubated with GANT-61 and TMZ (T98G: 296.9 ± 148.7–409.0%; U251: 261.5 ± 214.5–285.1%; GBM11: 213.4 ± 165.9–221.2%) or TMZ (T98G: 187.8 ± 124.1–295.5%; U251: 237.9 ± 138.4–246.7%; GBM11: 198.5 ± 191.5–221.2%) alone (Figure 5a). The results suggest an increase in catalase enzyme activity only in the U251 cells after the synergistic treatment. However, no significant changes in the antioxidant enzyme profile in the cell lines were observed (Figure 5b).

3.5 | Combined treatment of GANT-61 and TMZ induces cell death in GBM cell lines

TMZ exhibits the potential to activate apoptosis and autophagy events, leading the GBM cell lines to death (Sur, Srivnick, Patel, Ray, & Banik, 2005; Würstle et al., 2017). However, autophagy can present two different fates: cell survival or cell death (Filomeni et al., 2015; Wang et al., 2018). To evaluate if GANT-61 has the potential to increase cell death events promoted by TMZ, the membrane integrity and DNA fragmentation
FIGURE 3  Continued.
were assessed in the cell lines. All cell lines treated with TMZ concomitantly with GANT-61 presented a significant increase in PI incorporation, promoting the loss of membrane integrity (T98G: 58.9 ± 43.7–61.1%; U251: 47.1 ± 45.6–51.5%; GBM11: 35.1 ± 32.8–49.5%; Figure 7a). Considering DNA fragmentation as a final event of cell death, the T98G cell line also presented a higher percentual of cells presenting fragmented DNA (28.4 ± 26.0–39.9%), compared with U251 (12.1 ± 7.0–16.3%; Figure 7b). Meanwhile, GBM11 did not demonstrate any significant difference in cells with fragmented DNA under any of the experimental conditions. Thus, SHH inhibition seemed to sensitize heterogeneous human GBM cells (T98G and U251) to TMZ action and influence the resistant profile in others (GBM11).

4 | DISCUSSION

Glioblastoma is a deadly disease and the cure is, unfortunately, far from being found. Efforts are being carried out to develop new therapeutic methods to increase patient survival and improve the quality of life of patients with GBM. In this scenario, combination therapy is described as an essential element for this cancer’s treatment. To bring some hope to the search for new therapies for the treatment of GBM patients, in vitro assessments regarding the selective inhibition of the SHH pathway by GANT-61 on the TMZ effect potentiation were carried out.

In the last decade, TMZ has been the gold standard of treatment used in the clinic for GBM patients (Stupp et al., 2009, 2005). However, several patients are resistant to TMZ treatment due to the expression of the MGMT enzyme and MDR1 gene (Goellner et al., 2011; Munoz et al., 2015). In this study, U251 was the most sensitive cell line to TMZ, at 200 and 400 μmol/L when compared with T98G. In T98G, decreased cell viability was observed only at 72 hr. These results suggested that the cytotoxicity induced by TMZ for the U251 and T98G cell lines was concentration- and time-dependent, aligning with previous studies (Barciszewska, Gurda, Godowicz, Nowak, & Naskręt-Barciszewska, 2015). Moreover, this differential response to TMZ treatment may be due to the heterogeneity described for GBMs (Friedmann-Morvinski, 2014). It is interesting to note that GBM11 was the most resistant cell line, as no significant reduction in cell viability was observed, even after 72 hr of treatment. This may be due to the fact that this GBM cell line originated from a patient who relapsed after radiotherapy and TMZ treatment. In fact, a significant reduction in cell viability was noted when the U251 and T98G cell lines were treated concomitantly with GANT-61 and TMZ. The pharmacological combination of GANT-61 and TMZ sensitizes the glioma cells by enhancing the DNA damage effect and decreasing MGMT expression (Li et al., 2016). Thus, it is believed that SHH pathway inhibition by GANT-61 sensitized the assessed cell lines to TMZ. Moreover, it is important to note that the aberrant activation of the SHH pathway is involved with GBM resistance (Munoz et al., 2015).

GBM is known for its increased potential to migrate and infiltrate brain tissue, which represents the most challenging barrier to surgical resection (Chen et al., 2014). This ability is facilitated by recalling embryonic cells properties, through the activation of transcription factors that are responsible for the EMT phenomenon - a biological process where an epithelial cell acquires a mesenchymal cell profile with the ability to migrate, invade and resist chemotherapy (Ortensi et al., 2013). Among the EMT-related proteins overexpressed in GBM, vimentin - a major cytoskeletal proteins associated with cellular structure - has been associated with tumor progression and poor patient prognosis (Kalluri & Weinberg, 2009; Liu et al., 2015; Zhao et al., 2018). The concomitant incubation with GANT-61 and TMZ seemed to select therapy-resistant cells in both the T98G and U251 cell lines. As expected, these resistant cells presented higher GLI1 and vimentin expression. These expression profiles may be respectively associated to stemness and the EMT profiles may be respectively associated to stemness and the EMT negative prognostic factor linked to their positive effects on the CSC maintenance of an active pathway (Rossi et al., 2011).

The transcription factor of GLI1 is one of the main effectors of the SHH pathway, which is known for its importance in transcribing crucial genes, such as OCT-4, NANOG, and SOX-2, when translocated in the nucleus. The process observed during tumorigenesis entails high levels of GLI1 expression, which was observed through immunofluorescence in the study. High GLI1 expression in GBM appears to be an inherently negative prognostic factor linked to their positive effects on the CSC phenotype, which is considered the...
FIGURE 4  Continued.
main cause for tumor progression and therapeutic resistance (Boyle & Kochetkova, 2014; Lo et al., 2009). Moreover, SHH signaling through the GLI1–CSCs axis has been implicated in GBM’s resistance to TMZ, as CSCs preferentially activate the DNA damage checkpoint response and display an increased ability for DNA repair (Bao et al., 2006; Wu & Schöler, 2014). Since the SHH pathway reactivation can induce cellular proliferation and TMZ is an alkylating agent that promotes cell cycle arrest, this study investigated whether SHH inhibition could potentiate the TMZ cell cycle effects. As expected, TMZ alone was able to arrest the T98G cell cycle by inducing DNA damage. It is well established that cells with damaged genetic material are prevented from progressing in the cell cycle phase at the G1 and G2 checkpoints, thus becoming arrested and dying (Pietenpol & Stewart, 2002). With regard to GBM11, no effects on the cell cycle function after the treatment were observed, probably a consequence of the chemotherapeutic resistance of this cell line or because the cells were treated for only a short time. A previous study has highlighted a relationship between the degree of cell line sensitivity and TMZ’s capacity to induce cell cycle arrest, where the sensitive U373-MG cell line presented a higher proportion of

![FIGURE 5](image-url) TMZ alone or combined with GANT-61 induces ROS production. The T98G, U251, and GBM11 cell lines were incubated with 0.8% DMSO (CONTROL), GANT-61 20 μmol/L (GANT-61), TMZ 400 μmol/L (TMZ), and TMZ 400 μmol/L + GANT-61 20 μmol/L (TMZ + GANT-61) for 72 hr. (a) Reactive oxygen species levels. TMZ alone or combined with GANT-61 significantly increased ROS production in U251 and GBM11 cells, and TMZ combined with GANT-61 significantly increased ROS production in T98G cells. In (b), we can observe the catalase and GSH levels. There was no significant difference between the treatment conditions when evaluating the catalase and GSH activity. Both experiments were acquired by flow cytometry. Each graph is representative of three independent experiments, and the data is presented as the median ± min and max. An ANOVA one-way test followed by Tukey’s test for multiple comparisons was performed. ANOVA, analysis of variance; DCF, dichlorofluorescein; DMSO, dimethyl-sulfoxide; GBM, glioblastoma; GSH, glutathione; ROS, reactive oxygen species; TMZ, temozolomide

![FIGURE 4](image-url) TMZ alone or combined with GANT-61 induces cell cycle arrest in G2/M phase in the T98G cell line, but has no influence on Ki-67 proliferation marker expression. The T98G and GBM11 cell lines were incubated with 0.8% DMSO (CONTROL), GANT-61 20 μmol/L (GANT-61), TMZ 400 μmol/L (TMZ), and TMZ 400 μmol/L + GANT-61 20 μmol/L (TMZ + GANT-61) for 72 hr. In (a), we can observe the percentage of T98G and GBM11 cells in cell cycle phases G0/G1, S, and G2/M by flow cytometry. TMZ alone or combined with GANT-61 significantly decreased the percentual of T98G cells in G0/G1 phase, and significantly increased the percentual of T98G cells in the G2/M phase. No significant difference in cell density was observed for GBM11. (b) presents the expression profile of the Ki-67 protein for the T98G, U251, and GBM11 cell lines, and (c) presents the quantification of Ki-67 positive cells through immunofluorescence. There was no significant difference between the treatment conditions when evaluating Ki-67 expression. Each graph is representative of three independent experiments, and the data is displayed as the median ± min and max. An ANOVA one-way test followed by Tukey’s test for multiple comparisons was performed. ANOVA, analysis of variance; DMSO, dimethyl-sulfoxide; GBM, glioblastoma; TMZ, temozolomide. *p < .01; **p < .001
cells retained in G2/M than the more resistant cell line T98G (Kanzawa et al., 2003). In this study, GANT-61 alone or associated with TMZ did not induce or enhance cell cycle arrest even after 72 hr of treatment in GBM11 cells. This may be a consequence of its chemotherapeutic resistance (Kanzawa et al., 2003). Although it has been demonstrated that GANT-61 is able to slow down cell cycle progression by arresting both embryonal and alveolar rhabdomyosarcoma cells in the G0/G1 phase in a concentration-dependent manner (5–25 μmol/L; R. K. Srivastava et al., 2014), this effect was not observed herein probably due to the treatment being carried out only for 72 hr and with 20 μmol/L of GANT-61. Similarly, Ewing sarcoma cell treatment with 30 μmol/L of GANT-61 suppressed the S-phase transition, although the proportion of cells in the G0/G1 and G2/M phase did not increase (Matsumoto, Tabata, & Suzuki, 2014). In addition, no significant difference was observed between the Ki-67 expressions in the treatment groups evaluated herein, suggesting that GANT-61 and TMZ did not significantly enhance or reduce cell proliferation. Recently, it has been demonstrated that GANT-61 at 50 mg/kg decreases the Ki-67 expression in prostate cancer xenograft tumors (Gonnissen et al., 2016). These findings suggest that cell cycle arrest and Ki-67’s response to GANT-61 depends on its concentration and the tumor type, as no differences were
observed in cell proliferation in the GBM cell lines (Matsumoto et al., 2014).

The cellular oxidative system plays a crucial role during the regulation of the signaling pathways responsible for cell death and survival in cancer cases (Seyfrid et al., 2016). It is known that TMZ is able to activate the oxidative system via ROS production in GBM cells, although the TMZ resistance often observed in GBM has been associated to decreased ROS production (Seyfrid et al., 2016). Therefore, combined therapies have been used to increase ROS production, potentiating the TMZ effects (Yin et al., 2014). In recent years, oxidative stress has also been linked to autophagy induction through ROS production, which acts as a main intracellular signal that sustains the autophagy process (Filomeni et al., 2015). It is also well established that both ROS production and the SHH inhibitor GANT-61 have the ability to induce autophagy, an alternative mechanism activated during cellular stress that recycles damaged organelles, and, when present in higher levels, can lead to tumor cell death (Filomeni et al., 2015; Wang et al., 2018). Moreover, enhanced autophagy can induce cell death even in tumor cells (Wang et al., 2018). Although autophagy acts as a cell death mechanism, in some cases its can enhance TMZ resistance through degradation and recycling of unnecessary cellular components, as an adaptation mechanism to unfavorable microenvironmental conditions induced by cancer therapy (Jiaper, Furuta, Tanaka, Katabayashi & Nakada, 2018; Zou et al., 2019). Interestingly, GANT-61 concomitantly administered with TMZ increased ROS production in the T98G and U251 GBM cell lines, indicating that the SHH pathway inhibition could sensitize GBM cells to TMZ action by increasing the oxidative stress. Cell membranes are oxidative chemistry targets (Wang, Libardo, Angeles-Boza, & Pellois, 2017), and the ROS production observed herein seemed related to the increased membrane integrity loss after combining the treatment. This result is indicative of cell death, which was confirmed by the DNA fragmentation. Despite the differences observed in the level of ROS production, no significant

**FIGURE 7** The combined incubation of GANT-61 and TMZ induces loss of membrane integrity and DNA fragmentation in GBM cell lines. The T98G, U251, and GBM11 cell lines were incubated with 0.8% DMSO (CONTROL), GANT-61 20 μmol/L (GANT-61), TMZ 400 μmol/L (TMZ), and TMZ 400 μmol/L + GANT-61 20 μmol/L (TMZ + GANT-61) for 72 hr. (a) Presents the percentage of PI positive cells indicating loss of membrane integrity. TMZ alone increased the percentual of the T98G, U251, and GBM11 PI positive cells, which was statistically significant for T98G and U251. The combined treatment with GANT-61 significantly potentialized the TMZ effect on the T98G, U251, and GBM11 cells. (b) Presents the percentage of cells in the sub-G0/G1 phase indicating DNA fragmentation as a final cell death event. TMZ + GANT-61 significantly increased DNA fragmentation in the T98G and U251 cells. Both experiments were facilitated by flow cytometry. Each graph is representative of three independent experiments, and the data is displayed as the median ± min and max. An ANOVA one-way test followed by Tukey’s test for multiple comparisons was performed. ANOVA, analysis of variance; DMSO, dimethyl-sulfoxide; GBM, glioblastoma; TMZ, temozolomide. *p < .01; **p < .001; ***p < .0002; ****p < .0001
differences in the antioxidant enzyme activity was observed, but the combined treatment with GANT-61 and TMZ seems to have increased the catalase activity in the U251 cell line. It is possible that U251 can activate antioxidant mechanisms against cellular oxidation. In view of these results, this study assessed whether enhanced ROS production after the combined treatment could influence GANT-61-induced autophagy. An increased AVO production after the treatment was observed in all cell lines, especially the T98G cell line. Moreover, increased Beclin-1 expression was also observed in the T98G and GBM11 cell lines after combined treatment. Interestingly, the Beclin-1 expression on U251 cell line was statistically significant in the TMZ-only treatment. These findings indicate that ROS production after the GANT-61 treatment associated to TMZ has the potential to induce autophagy. It is interesting to note that SHH pathway activation has been linked to reduced oxidative damage in cardiomyoblast cells, and its inhibition by cyclopamine seems to increase ROS production in these cells (Zhang, Qiao, Liu, & Ma, 2018).

In conclusion, in this in vitro study we demonstrated the heterogeneity between different human GBM cell lines. Treatment using TMZ combined with GANT-61 can interfere in the oxidative stress balance in all GBMs. On the other hand, autophagy mechanisms demonstrated two different fates for these tumor cells. The most sensitive GBM presented the G2/M cell cycle arrest followed by autophagy and relevant cell death. Meanwhile, other GBM cell line demonstrated alteration in molecules associated with EMT phenotype and antioxidant profile, resulting in an intermediary level of cell death. Furthermore, the most resistant GBM physiologically acquired TMZ resistance before we established the primary culture. Then, the autophagy observed in these cells could have been associated with survival (Figure 8). In conclusion, TMZ resistance can be overcome with SHH pathway inhibition. However, personalized medicine should be considered as the therapeutic approach once TMZ resistance is multifactorial considering the GBM’s phenotype.

These findings highlight the importance of studying SHH signaling in GBM during TMZ treatment. EMT, epithelial–mesenchymal transition; GBM, glioblastoma; ROS, reactive oxygen species; SHH, Sonic hedgehog.
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Authors elect to not share data.

ORCID
Tania C. L. de S. Spohr http://orcid.org/0000-0001-9502-2314

REFERENCES


