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Research Article

Molecular mechanism of GANT61 combined with doxorubicin in the treatment of gliomas based on network pharmacology

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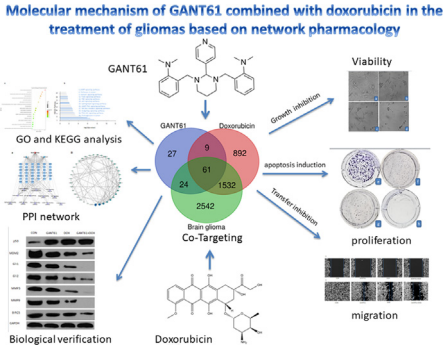
GRAPHICAL ABSTRACT



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ABSTRACT

Background: Gliomas are common malignant intracranial tumors. Efficacious targeted therapy against gliomas is lacking.
Results: GANT61 combined with the chemotherapy drug doxorubicin for treatment of glioma (LN-229) cells, and the effect of their combination, was tested. The molecular mechanism was explored by target prediction, along with functional analysis using the Gene Ontology (GO) database, enrichment analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, construction of protein–protein interaction (PPI) networks, and protein expression. Combination of GANT61 plus doxorubicin could inhibit the growth of LN-229 cells effectively. Wound-healing data and expression of migration proteins related to epithelial-to-mesenchymal transition showed that this combination could inhibit the migration of LN-229 cells. Sixty-one targets of drug and disease intersected. Functional analysis revealed negative regulation of apoptosis, positive regulation of cell proliferation, and other biological processes related to apoptosis and proliferation. Pathway-enrichment analysis showed drug combination to be related to the cyclic adenosine monophosphate signaling pathway, pathways in cancer, and Hedgehog signaling pathway. Measurement of expression of several proteins related to these pathways revealed expression of BIRC5, GLi1 and GLi2, MMP3 and MMP9 proteins to decrease, and expression of MDM2 and P53 proteins to decrease and increase, respectively.

Abbreviations: ADRB2, adrenergic, beta-2-; BIRC5, baculoviral IAP repeat containing 5; Camp, Cyclic adenosine monophosphate; CDH1, Cadherin 1; DRD1, Recombinant Dopamine Receptor D1; EMT, Epithelial-Mesenchymal Transition; EP300, E1A binding protein p300; GO, Gene Ontology; JAK2, Janus kinase 2; KEGG, Kyoto Encyclopedia of Genes and Genomes; MAPK8, Recombinant Mitogen Activated Protein Kinase 8; MDM2, murine double minute2; MMP9, Matrix metalloproteinase-9; PPI, protein protein interaction; TLR4, Toll-like receptor 4; ZEB1, Zinc Finger E-Box Binding Homeobox 1.

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Conclusions: This study provides a: (a) new direction for targeted therapy of gliomas; (b) theoretical basis for drug research and molecular-mechanism research on gliomas.

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1. Introduction

Gliomas are brain tumors that originate in glial cells. They have high growth rate, recurrence, and mortality [1,2,3,4,5]. First-line treatment for gliomas is surgery combined with radiotherapy, chemotherapy, and immunotherapy. Doxorubicin is one of the most efficacious drugs against gliomas. However, irrespective of the type of treatment, there are limitations, such as drug resistance and toxic side-effects. There is an urgent need to obtain new concepts for the drugs used to treat gliomas [6,7].

"Network pharmacology" (NP) integrates information science and systematic medicine. It has been used to study the molecular mechanisms of traditional Chinese medicine formulations in recent years. Through establishment of a multidimensional data network, NP can reveal the "drug–target–disease" relationship, predict the mechanism of action, as well as evaluate the efficacy and adverse reactions, of a drug [8]. In this way, highly efficacious and low-toxicity drugs can be discovered. For example, NP has been employed to explore the anti-aging function and molecular mechanism of *Radix astragali* [9] and reveal the mechanism of action of scopoletin against non-small-cell lung cancer [10].

As a specific blocker of the transcription factor Gli in the Hedgehog signaling pathway, GANT61 can act directly on Gli 1/2. GANT61 can specifically block activation of the downstream target genes mediated by Gli 1/2, thereby inhibiting the proliferation of tumor cells [11]. It has been demonstrated that GANT61 has an anti-tumor role in the treatment of triple-negative breast cancer [12], T-cell lymphoma [13], and cervical cancer [14]. Some studies have found that Gli1 overexpression is closely related to tumor chemoresistance, and GANT61 can block Gli 1 specifically, thereby improving the chemosensitivity of malignant tumor cells and increasing the efficacy of chemotherapy drugs. For example, GANT61 not only reduces the growth of triple-negative breast cancer cells, it also enhances the anti-tumor activity of paclitaxel in these cells [12]. In addition, It has been reported that the anticancer effect of GANT61 is related to the phosphoinositide 3-kinase/protein kinase B (PI3K/AKT) signaling pathway [15]. A new method for cancer treatment could be the combined use of drugs [16].

In view of the side-effects of doxorubicin and the resistance of gliomas to the actions of drugs, we used GANT61 combined with doxorubicin to study glioma treatment. Use of NP to explore the molecular mechanisms of drug combinations could provide new targets for glioma treatment.

2. Materials and methods

2.1. Ethical approval of the study protocol

We used a cell line, so ethical approval was not required to carry out this study.

2.2. Materials

A human-brain glioblastoma cell line (LN-229) was purchased from iCell Bioscience (Shanghai, China). Doxorubicin, GANT61, and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution were obtained from Sigma–Aldrich (Saint Louis, MO, USA). RPMI 1640 medium, fetal bovine serum, streptomycin, and Matrigel™ were purchased from Gibco (Grand Island, NY, USA). Paraformaldehyde (4%), sodium chloride, potassium chloride, potassium dihydrogen phosphate, phenol, isopropanol, and 30% hydrogen peroxide were supplied by Sinopharm Chemical Reagents (Beijing, China). BCIP/NBT kit, Citrate buffer was purchased from Servicebio (Beijing, China). Hematoxylin staining solution, secondary antibodies, horseradish peroxidase-labeled goat anti-rabbit secondary antibody, histochemical kit, and 3,3′-Diaminobenzidine were obtained from Beyotime Institute of Biotechnology (Beijing, China).

2.3. Observation of cell morphology

LN-229 cells in the logarithmic-growth phase were diluted to 1×10^4 cells/mL. One-milliliter of this suspension was used to inoculate a six-well plate (1×10^4 cells/well), followed by cell culture in an incubator in an atmosphere of 5% carbon dioxide at 37 °C. After confluence, a control group and experimental group were created. Complete medium (2 mL) was added to cells in the control group. In the experimental group, 2 mL of medium containing GANT61 (80 μmol/L), doxorubicin (1 μmol/L), or GANT61 (80 μmol/L) + doxorubicin (1 μmol/L), respectively, was added to cells. Then, the cells were cultured at 37 °C in an atmosphere of 5% CO_2 for 48 h. The morphology of LN-229 cells was observed and recorded by an inverted microscope.

2.4. Wound healing

LN-229 cells in the logarithmic growth phase were removed and counted using a microscope. 1×10^4 cells were inoculated in each well. Then, they were inoculated with 1 mL of drug in each well and cultured in an atmosphere of 5% CO_2 at 37 °C. When cells had reached 80%–90% confluence, a line perpendicular to the horizontal line behind the six-well plate was drawn. After scratching of cells, they were viewed under a microscope and photographed. The scratch width of the original cell was recorded as 0 h. In the control group, only 10% serum medium was added. In the experimental group, 10% serum medium containing GANT61 (80 μmol/L), doxorubicin (1 μmol/L), or GANT61 (80 μmol/L) + doxorubicin (1 μmol/L), respectively, was added, followed by culture in an atmosphere of 5% CO_2 at 37 °C for 48 h. At the end of culture, the scratch width of cells at 48 h vs the scratch width of cells at 0 h.

2.5. MTT assay

LN-229 cells in the logarithmic growth phase were selected. After counting under a microscope, 5×10^3 cells/well was inoculated into 96-well plates. The 96-well plates were cultured in an atmosphere of 5% CO_2. After cells had adhered to the wall completely, the drug was administered. The groups were GANT61 (80 μmol/L), doxorubicin (1 μmol/L), and GANT61 (80 μmol/L) + doxorubicin (1 μmol/L). The control group did not have drug loading, and the well without cell were set as blank well. Six parallel

wells were set in each group and cultured overnight in an atmosphere of 5% CO_2 at 37 °C for 48 h. Then, 20 μL of MTT solution (5 mg/mL) was added to each well followed by culture for 4 h. The culture medium was discarded, and 150 μL of dimethyl sulfoxide was added. The solution was well mixed until the purple–brown precipitate had dissolved completely. The optical density (OD) of each well was measured at 490 nm. Cell viability was calculated according to the following formula:

Cell survival (%) = ($OD_{Experimental\ group} - OD_{Zero\ adjustment\ hole}$/ $OD_{Control\ group} - OD_{Zero\ adjustment\ hole}$) × 100%.

2.6. Colony-formation assay

Cells were added to six-well plate (1×10^3 cells/well) following transfection for 2 weeks. 2 mL of complete medium was added to the control group, 2 mL of medium containing GANT61 (80 μmol/L), doxorubicin (1 μmol/L), or GANT61 (80 μmol/L) + doxorubicin (1 μmol/L) was added cells, respectively. Colonies were fixed with 100% methanol at room temperature for 20 min and stained with 0.1 % crystal violet (Sigma-Aldrich; Merck KGaA) at 25 °C for 30 min. The total number of visible colonies was imaged and counted using a light microscope (magnification, ×100). All experiments were repeated three times. Photographs were taken using ImageJ (US National Institutes of Health, Bethesda, MD, USA). The colony-formation rate was calculated using the following formula:

Colony-formation rate (%) = number of colonies in the experimental group/number of colonies in the control group × 100%.

2.7. Target prediction of drug combinations against glioma

We collected basic information and predicted targets of GANT61 and doxorubicin through databases: PubChem (https://pubchem.ncbi.nlm.nih.gov/), Similarity Ensemble Approach (SEA) Search Server (https://sea.bkslab.org/), and Superpred (https://prediction.charite.de/). The UniProt (www.uniprot.org/) database was employed to search for the protein name of the drug and obtain the gene name corresponding to the predicted target. Then, we obtained the glioma gene in the GeneCards (www.genecards.org/) database. We used the "SUMIF" function in Excel™ (Microsoft, Redmond, WA, USA) to find the intersections between the related target genes and candidate target genes of GANT61 and doxorubicin. The three common target genes were used as potential targets for the combination of GANT61 and doxorubicin against glioma. OmicShare (www.omicshare.com/) was used to draw a Venn diagram of the three common targets.

2.8. Use of the gene Ontology (GO) database to show functional enrichment

GO focuses on the function of genes and gene products. We carried out GO on the intersection targets using the GO database (http://geneontology.org/). We screened out 20 biological processes and drew a "bubble" diagram in which red denoted functional enrichment.

2.9. Pathway enrichment using the Kyoto Encyclopedia of genes and Genomes (KEGG) database

The KEGG database deals with genomes, biological pathways, diseases, drugs, and chemical substances. Analyses of pathway enrichment were done on the intersection targets in Database for Annotation, Visualization and Integrated Discovery (DAVID). $P < 0.05$ was used as the screening condition for a significant difference. Screened items were drawn into a bar graph, and then the KEGG items related to signaling pathways were screened out and marked blue. Information on targets, diseases, and drugs related to signaling pathways was imported into Cytoscape 3.6.0 (https://cytoscape.org/) to construct and visualize the network topology of disease–drug–target–signaling pathways.

2.10. Protein–protein interaction (PPI)

The potential targets of GANT61 combined with doxorubicin against glioma were imported into the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (https://string-db.org/). The species was set as *Homo sapiens*. The interactions between target proteins were retrieved and imported into Cytoscape 3.6.0. Network analysis was undertaken using the "Network Analyzer" tool, and the "degree" value and "betweenness centrality" value were calculated. The PPI network was drawn by Cytoscape 3.6.0, and shown as a diagram.

2.11. Western blotting

LN-229 cells were cultured in culture medium containing GANT61 (80 μmol/L), doxorubicin (1 μmol/L), or GANT61 (80 μmol/L) + doxorubicin (1 μmol/L), respectively. Cells in the control group were cultured in complete culture medium. Cells were lysed by a homogenizer. The lysed solution was transferred to an Eppendorf™ tube. The liquid was centrifuged (10,000×*g* for 5 min at 4 °C), and the supernatant removed and stored at −20 °C.

The sample was heated in boiling water for 5–10 min to denature the protein fully. 10% ammonium persulfate and 10 ml 10% polyacrylamide lower layer separation glue; Glue; 6 mL 5% polyacrylamide concentrate glue after pouring glue to make the upper concentrated glue; After loading the sample, the lower glue board was installed in an electrophoresis tank. Electrophoresis buffer was added. After electrophoresis had been completed, the rubber plate was removed. The rotary film clip was opened, and a polyvinylidene difluoride (PVDF) film placed on the glue. The rotary film clip was closed, and rotary film buffer added to transfer the film. After that, the PVDF membranes was washed thrice with TBST (Tris-buffered saline + Tween 20). The PVDF membrane was sealed with 5% skimmed milk powder plus TBST. After removal, primary antibody was added followed by incubation at 4 °C overnight. The PVDF membrane was removed and washed thrice with TBST. Secondary antibody was added followed by incubation for 1 h. An electrochemiluminescence western blotting kit (BCIP/NBT) was used to detect target bands. ImageJ was used for quantification.

2.12. Statistical analyses

Data were processed using SPSS 25.0 (IBM, Armonk, NY, USA). Data are the mean ± standard deviation. Data were analyzed using the Student's *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. Drug combination inhibits the growth of LN-229 cells

To understand the effect of drug combinations, we tested the effect of GANT61 combined with doxorubicin on the growth, proliferation, and apoptosis of LN-229 cells.

LN-229 cells were treated with GANT61 (80 μmol/L), doxorubicin (1 μmol/L), or GANT61 (80 μmol/L) + doxorubicin (1 μmol/L), respectively. An inverted microscope was employed to observe changes in morphology (Fig. 1a–d). Forty-eight hours after treatment, cells in the control group grew faster, and their number covered the entire field of view, and the morphology was regular. Compared with the control group, the number of adherent cells in the GANT61 group decreased slightly, and the cells shrank and

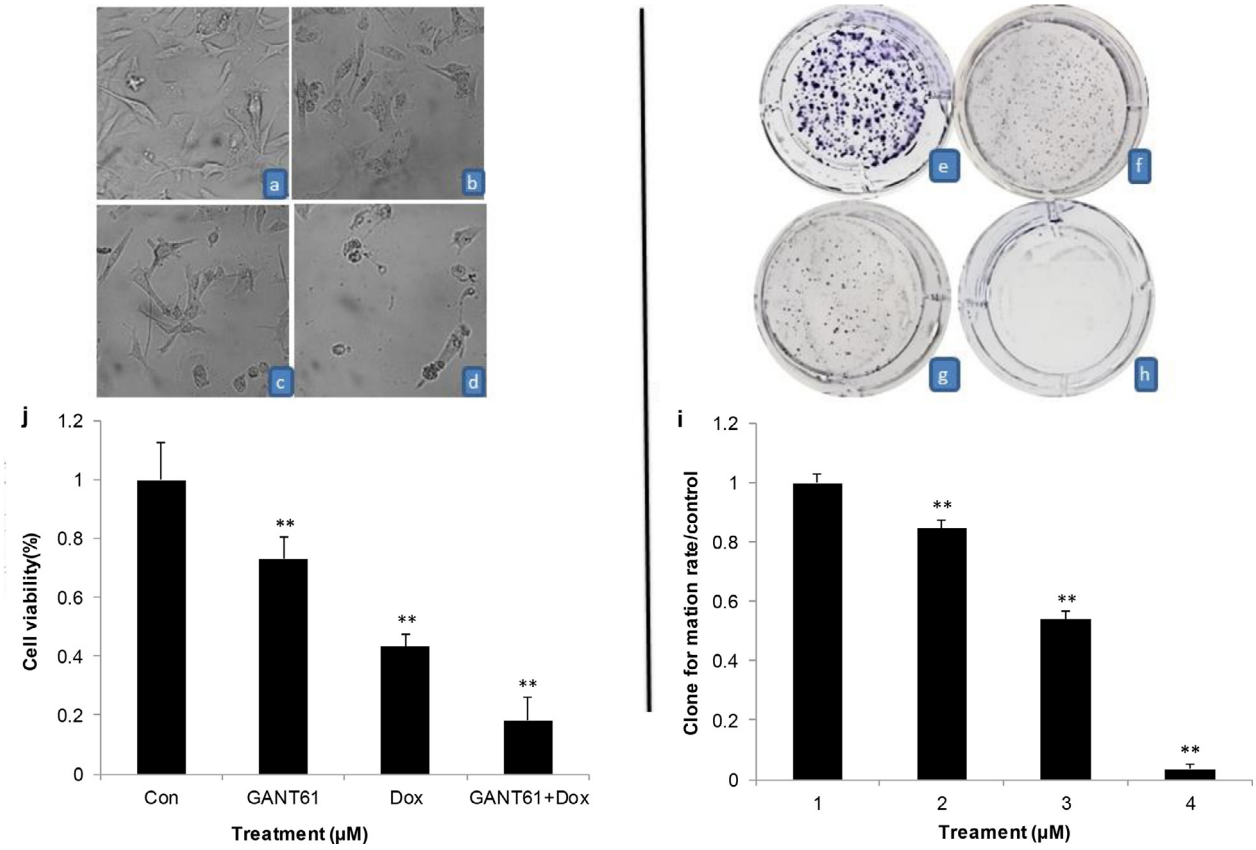

Fig. 1. Effect of GANT61 combined with doxorubicin on the growth of LN-229 cells. (a–d) Morphology of LN-229 cells; (e–i) Proliferation of LN-229 cells; (a, e: control group; b, f: GANT61 group; c, g: doxorubicin group; d, h: GANT61+ doxorubicin group); j. Viability of LN-229 cells. Compared with the control group, **p < 0.01.

gradually became round. Compared with the control group, the number of adherent cells in the doxorubicin group decreased significantly. Compared with the control group, the cells in the GANT61+ doxorubicin group showed obvious shrinkage, circularity, and shedding, and the effect was more obvious than that seen with GANT61 alone or doxorubicin alone. These data showed that a combination of GANT61 and doxorubicin could inhibit the viability of LN-229 cells.

Next, we explored the colony-forming ability of LN-229 cells after combination treatment. Compared with the control group, the number of colonies formed in the GANT61 group, doxorubicin group, and GANT61 + doxorubicin group was reduced significantly (Fig. 1e–i). The order of colony formation from high to low was control group > GANT61 group > doxorubicin group > GANT61 + doxorubicin group. Compared with the control group, the percentage of colonies formed in the GANT61 group, doxorubicin group, and GANT61 + doxorubicin group was 85 ± 2.69%, 54 ± 3.01%, and 4 ± 1.34%, respectively, and the difference was significant (P < 0.01 for all). These data indicated that, at the concentrations tested, GANT61 and doxorubicin inhibited the proliferation of LN-229 cells significantly, and that the combined effect of GANT61 and doxorubicin on cell proliferation was stronger than when the two were used singly.

Next, we measured the survival of LN-229 cells by the MTT assay. GANT61 and doxorubicin could inhibit the survival of LN-229 cells (Fig. 1j). Compared with the control group, cell survival in the GANT61 (80 μmol/L) group, doxorubicin group (1 μmol/L) and GANT61 + doxorubicin group decreased to 73 ± 8.19%, 43 ± 4.73%, and 18 ± 8.00%, respectively, and the difference was significant (P < 0.01). Upon combination of GANT61 and doxorubicin, the inhibitory effect on LN-229 cells was more obvious than that when used alone, and most of the cells died. These data showed that, within the tested concentration range, GANT61 and doxorubicin could inhibit the survival of LN-229 cells effectively.

These results suggested that GANT61 combined with doxorubicin could inhibit LN-229 cells significantly, and could have a significant anti-glioma effect.

3.2. Drug combination inhibits the migration ability of LN-229 cells

Cell migration is a major reason for the poor treatment effect and high mortality of cancer patients [17]. The cell-scratch experiment was used to detect the effect of drug combination on the migration ability of LN-229 cells (Fig. 2). Compared with 0 h (Fig. 2a), scratches on the cells in the four treatment groups in Fig. 2b healed 48 h after culture, indicating that the cells had migrated during culture. Cells in the control group had healed fully. Compared with the control group, the degree of healing in the GANT61 group, doxorubicin group, and GANT61+ doxorubicin group decreased successively, and the degree of wound healing of the GANT61+ doxorubicin group was the weakest. Hence, after drug combination, the migration ability of LN-229 cells was inhibited strongly.

The phenomenon of "cadherin conversion" is a key feature of the epithelial-to-mesenchymal transition (EMT) of ovarian epithelial tumors. That is, E-cadherin expression is downregulated and N-cadherin expression is upregulated, which has been demonstrated to be related to cancer occurrence [18]. E-cadherin expression is regulated by transcription factors (e.g., Snail, Slug, Twist, ZEB1, ZEB2) which inhibit E-cadherin expression by binding to the E-box sequence in the CDH1 promoter region, which leads

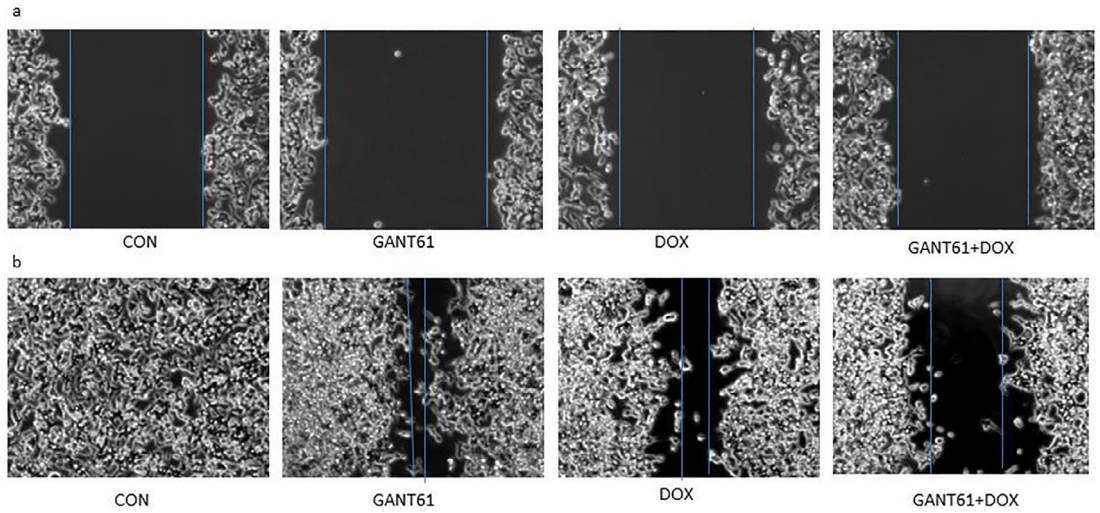

Fig. 2. Effect of GANT61 combined with doxorubicin on the migration of LN-229 cells. (a) Cells were cultured for 0 h. (b) Cells were cultured for 48 h.

to EMT [19]. We measured the protein expression of N-cadherin, E-cadherin, Twist1, and ZEB1 to further demonstrate the migration ability of NL-299 cells after combined-drug treatment. Compared with the control group, expression of N-cadherin protein in the administration group decreased significantly, and expression of Twist1 protein and ZEB1 protein in the doxorubicin group and GANT61+ doxorubicin group decreased (Fig. 3). Expression of E-cadherin protein increased in the doxorubicin group and GANT61 + doxorubicin group. These differences were significant. Hence, GANT61 combined with doxorubicin had a significant effect upon glioma treatment.

3.3. Common targets in GANT61, doxorubicin, and glioma

Using databases to collect drug-related information, we obtained the related structures of GANT61 and doxorubicin (Fig. 4a, b). The number of extracted targets of GANT61 and doxorubicin was 121 and 2494, respectively (Fig. 4c). A total of 4159 targets were related to glioma. The intersection of GANT61-, doxorubicin-, and glioma-related targets yielded 61 targets. Hence, a combination of GANT61 and doxorubicin in treatment of glioma was through multi-target interactions.

3.4. Targets of drug combination using the GO database

GO has been used widely in bioinformatics analysis in recent years [20]. It is used to annotate genes of various species, and describe their function. This strategy lays the foundation for the conversion and mining of data [21].

The GO database showed the top-20 biological processes to include "signaling pathways involved in regulation of cerebellar granule cell proliferation", "JUN phosphorylation", "negative regulation of apoptotic process", "positive regulation of cell proliferation", "regulation of signal transduction by p53 class mediator", "inflammatory response" and other tumor-related biological processes (Fig. 5a). It appeared that GANT61 combined with

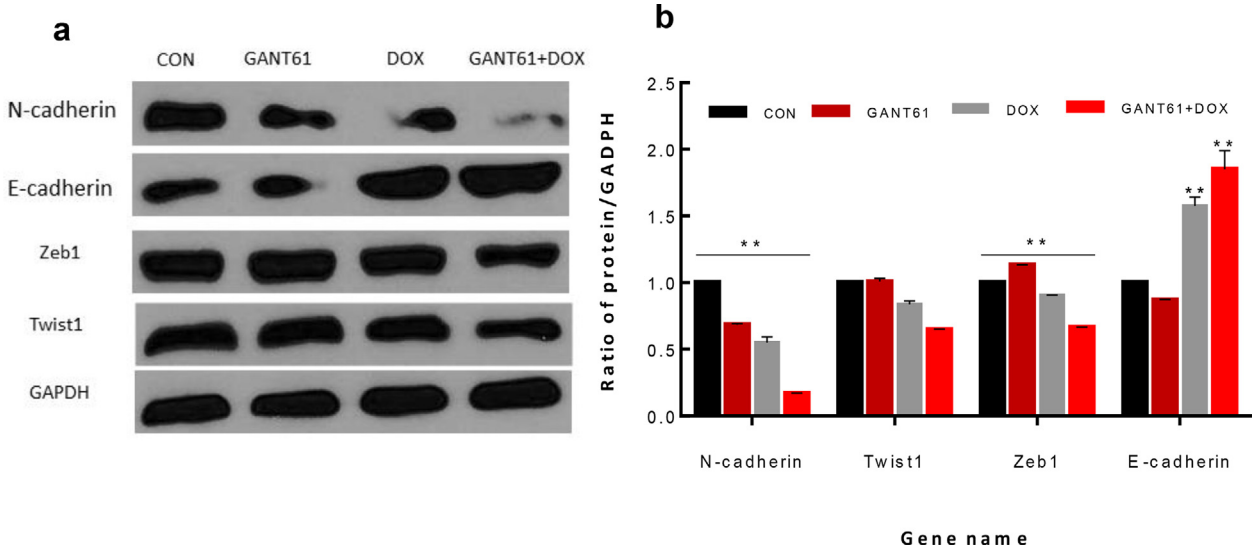

Fig. 3. Effect of GANT61 combined with doxorubicin on expression of cell migration-related proteins. (a) Expression of N-cadherin, E-cadherin, Zeb1, Twist1, and GAPDH as measured by western blotting. (b) Compared with GAPDH, the ratio of protein expression of N-cadherin, E-cadherin, Zeb1, Twist1 and GAPDH as measured by western blotting. Compared with the control group, $^{**}p < 0.01$.

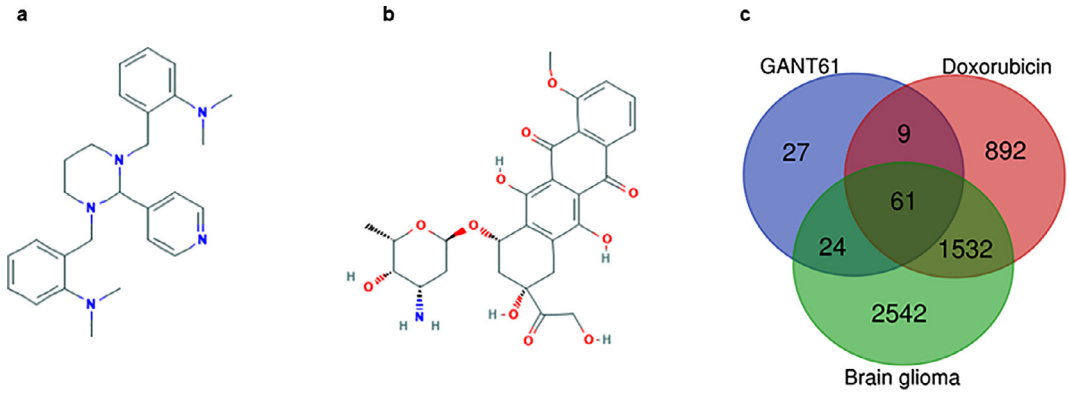

Fig. 4. Structure of GANT61, doxorubicin, and a Venn diagram showing targets. (a) Structure of GANT61. (b) Structure of doxorubicin. (c) Venn diagram of GANT61 combined with doxorubicin on migration-related targets.

doxorubicin could be used to treat glioma by regulation of various complex biological processes.

3.5. Targets of drug combination using the KEGG database

Pathway-enrichment analysis was done on the targets of GANT61 combined with doxorubicin against glioma using the KEGG database. The main related signaling pathways were "cAMP signaling pathway", "Pathways in cancer", "TNF signaling pathway", "p53 signaling pathway", and "Hedgehog signaling pathway" (Fig. 5b). These pathways are closely related to cancer. For example, wild-type P53 (tumor suppressor gene) can induce DNA-damage repair, growth arrest, and apoptosis [22]; tumor necrosis factor (TNF) can damage tumor cells and kill them [23]; the hedgehog signaling pathway improves the self-renewal, invasion, and metastasis of tumor cells [24].

3.6. Construction of a PPI network of target proteins and pathways

Cytoscape was used to draw the "compound–target gene–pathway" topology network diagram (Fig. 6a). Statistical analyses of 12 signaling pathways and 39 target genes revealed that GANT61 combined with doxorubicin regulated apoptosis, metastasis, and cell proliferation by acting on multiple targets and, finally, induced apoptosis of glioma cells.

Using STRING as the background network database, the data of interactions of 39 target proteins were obtained, and a PPI network was drawn. In the PPI network (Fig. 6b), 39 nodes interacted through 238 edges. The circular nodes represent target genes, and the straight lines between nodes indicate that there is interaction between the two linked proteins. The thicker the lines, the stronger is the interaction. The larger the node and the darker the color, the greater is the degree value of the target gene. Among them, the nodes of the targets MMP9, MAPK8, MDM2, DRD1, TLR4, EP300, JAK2, ADRB2, and MMP3 were relatively large, indicating that the degree value was relatively large. Hence, these targets were predicted to have important roles in GANT61 combined with doxorubicin against gliomas.

3.7. Expression of NP-related proteins

Based on the biological processes, signaling pathways, and targets obtained in the process described above, we screened some proteins for verification. According to western blotting (Fig. 7), compared with the control group, expression of BIRC5, GLI1, GLI2, MDM2, MMP3, and MMP9 proteins in the cells of each treatment group decreased significantly, and protein expression of P53 in the treatment group increased in a significant manner.

4. Discussion

Gliomas are malignant intracranial tumors with high incidence, high mortality, strong invasiveness, and carrying a poor prognosis [25]. With the rapid development of molecular biology, increasing attention has been paid to application of genetic detection in tumors, and precision therapy has elicited considerable benefits to patients. However, there are few clinical data on the targeted therapy of gliomas [26].

GANT61 can interfere with the binding of GLi to highly conserved DNA sequences in the hedgehog signaling pathway. We discovered, using the KEGG database, that the hedgehog signaling pathway was involved in the manner by which GANT61 combined with doxorubicin affects gliomas. Also, analyses of PPI networks showed that GLi protein is involved in this process.

Studies have shown that MMP3 and MMP9 can enhance the infiltration and metastasizing ability of tumor cells, and can be used as markers of gliomas [27]. MMP9 expression is related to the grade and survival of gliomas, and is an independent prognostic factor for primary gliomas [28]. MMP3 can degrade types III, IV, and V collagen, fibronectin, and laminin [29]. Analyses of PPI networks showed that the nodes for MMP3 protein and MMP9 protein were large, and that they could play a major role in GANT61 combined with doxorubicin against gliomas. Expression of MMP3 protein and MMP9 protein decreased, indicating that GANT61 combined with doxorubicin could inhibit expression of MMP3 protein and MMP9 protein, which would affect gliomas.

Analyses of the KEGG database showed that the cyclic adenosine monophosphate (cAMP) signaling pathway is an important signaling pathway. The invasion and metastasis of glioma cells is related to the cAMP pathway [30]. cAMP is an important intracellular regulatory factor. It can inhibit the proliferation of glioma cells and induce their differentiation and apoptosis [31,32,33]. cAMP catalyzes the production of adenylate cyclase by adenosine triphosphate, which participates in various extracellular stimuli (e.g., hormones, growth factors, neurotransmitters). G protein-coupled receptors activate adenylate cyclase and increase intracellular levels of cAMP, thereby regulating cell proliferation and apoptosis [34,35]. Functional analyses using the GO database revealed the adenosine receptor signaling pathway, adenylate cyclase-activating G-protein coupled receptor signaling

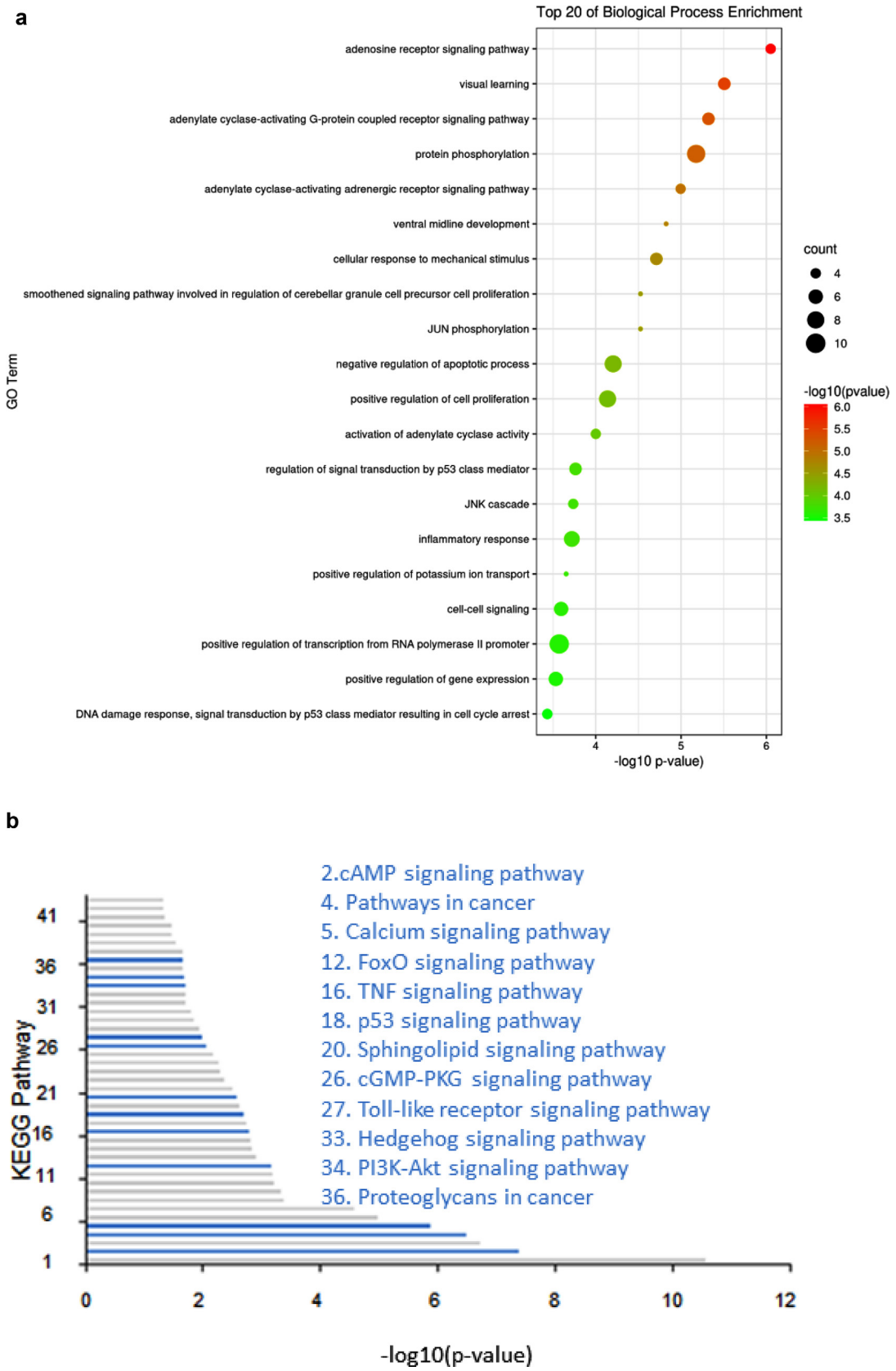

Fig. 5. Analyses of the potential targets and pathways for GANT61 combined with doxorubicin for glioma treatment. (a). GO analysis of potential targets; (b). KEGG analysis of pathway.

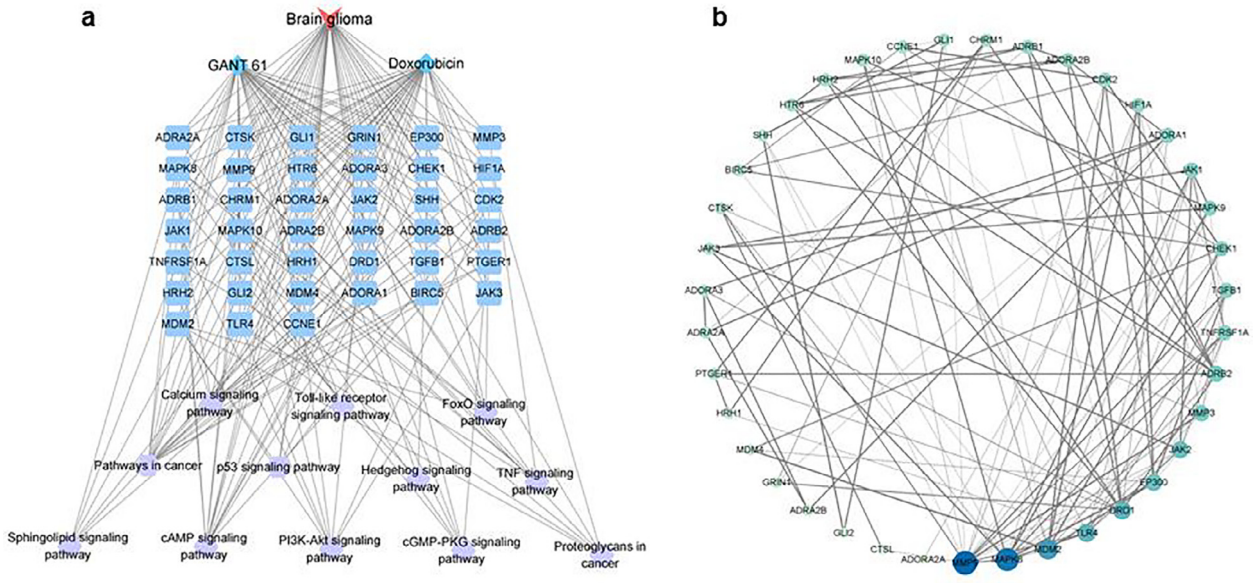

Fig. 6. Interaction of pathways, targets and diseases. (a) Interaction of pathways–targets–diseases. (b) Interaction of targets – targets.

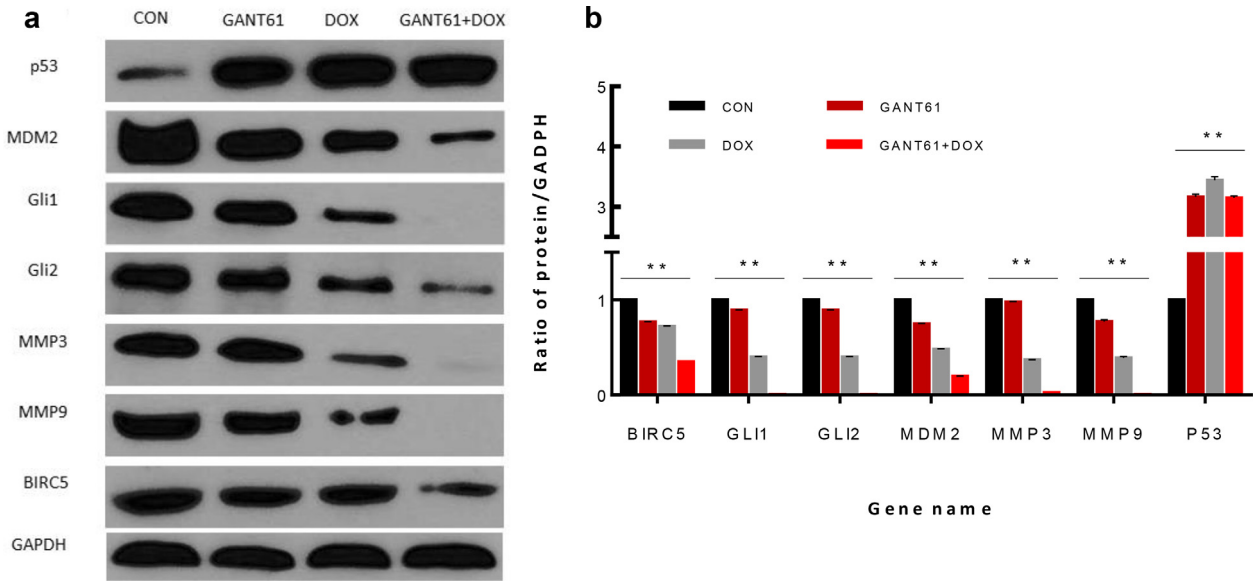

Fig. 7. Expression of protein related to drug combinations. (a) Expression of p53, MDM2, Gli1, Gli2, MMP3, MMP9, BIRC5 and GAPDH as measured by western blotting. (b) Compared with GAPDH, the ratio of protein expression of p53, MDM2, Gli1, Gli2, MMP3, MMP9, BIRC5 and GAPDH as measured by western blotting. Compared with the control group, $^{**}p < 0.01$.

pathway, and other biological processes related to adenylate cyclase. These processes are closely related to the cAMP signaling pathway.

5. Conclusions

Our study demonstrated that GANT61 combined with doxorubicin could inhibit glioma cells significantly. Through NP we found that, in addition to the hedgehog signaling pathway and related GLi proteins, other targets (MMP3, MMP9) and other pathways (cAMP signaling pathway) could provide new targets for glioma treatment.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] Sun H, Yang B, Zhang H, et al. RRM2 is a potential prognostic biomarker with functional significance in glioma. Int J Biol Sci 2019;15(3):533–43. https://doi.org/10.7150/ijbs.30114.
[2] Huang J, Chen H, Wei Q, et al. Downregulation of LKB1 promotes tumor progression and predicts unfavorable prognosis in patients with glioma. Oncol Lett, 2017;13(3):1688–1694. https://doi.org/10.3892/ol.2017.5631. PMid: 28454310.
[3] Liu Y, Mei L, Xu C, et al. Dual receptor recognizing cell penetrating peptide for selective targeting, efficient intratumoral diffusion and synthesized anti-glioma therapy. Theranostics 2016;6(2):177–91. https://doi.org/10.7150/thno.13532. PMid: 26877777.
[4] Joshi S, Cooke JRN, Chan DKW, et al. Liposome size and charge optimization for intraarterial delivery to gliomas. Drug Deliv Translat Res 2016;6(3):225–33. https://doi.org/10.1007/s13346-016-0294-y. PMid: 27091339.
[5] Zong T, Mei L, Gao H, et al. Synergistic dual-ligand doxorubicin liposomes improve targeting and therapeutic efficacy of brain glioma in animals. Mol Pharm 2014;11(7):2346–57. https://doi.org/10.1021/mp500057n. PMid: 24893333.
[6] Wang J, Shi M, Ling R, et al. Adjuvant chemotherapy and radiotherapy in triple-negative breast carcinoma: a prospective randomized controlled multi-center trial. Radiother Oncol 2011;100(2):200–4. https://doi.org/10.1016/j.radonc.2011.07.007. PMid: 21852010.
[7] Kiyokawa J, Wakimoto H. Preclinical and clinical development of oncolytic adenovirus for the treatment of malignant glioma. Oncolytic virotherapy 2019;8:27–37. https://doi.org/10.2147/OV.S196403. PMid: 31750274.
[8] Zhang Y, Cai B, Zhang Z. Application progress of network pharmacology in traditional chinese medicine research. J Nanjing Univ Tradit Chinese Med 2021;37(01):156–60. https://doi.org/10.14148/j.issn.1672-0482.2021.0156.
[9] Gong P, Wang D, Cui D, et al. Anti-aging function and molecular mechanism of *Radix Astragali* and *Radix Astragali* preparata via network pharmacology and PI3K/Akt signaling pathway. Phytomedicine 2021;84:153509. https://doi.org/10.1016/j.phymed.2021.153509. PMid: 33636579.
[10] Yuan C, Wang M-H, Wang F, et al. Network pharmacology and molecular docking reveal the mechanism of Scopoletin against non-small cell lung cancer. Life Sci 2021;270:119105. https://doi.org/10.1016/j.lfs.2021.119105. PMid: 33497736.
[11] Chaturvedi NK, Kling MJ, Coulter DW, et al. Improved therapy for medulloblastoma: targeting hedgehog and PI3K-mTOR signaling pathways in combination with chemotherapy. Oncotarget, 2018;9(24):16619–16633. https://doi.org/10.18632/oncotarget.24618, PMid: 29682173.
[12] Koike Y, Ohta Y, Saitoh W, et al. Anti-cell growth and anti-cancer stem cell activities of the non-canonical hedgehog inhibitor GANT61 in triple-negative breast cancer cells. Breast Cancer 2017;24(5):683–93. https://doi.org/10.1007/s12282-017-0757-0. PMid: 28144905.
[13] Geng L, Lu K, Li P, et al. GLI1 inhibitor GANT61 exhibits antitumor efficacy in T-cell lymphoma cells through down-regulation of p-STAT3 and SOCS3. Oncotarget, 2017;8(30):48701–10. https://doi.org/10.18632/oncotarget.9792, PMid: 27275540.
[14] Li L, Chen H, Li P, et al. Effect of GANT61 on the proliferation, migration, and invasion ability of Hela cells. Med J Wuhan Univ 2018;39(03):394–8. https://doi.org/10.14188/j.1671-8852.2017.0780.
[15] Borah A, Pillai SC, Rochani AK, et al. GANT61 and curcumin-loaded PLGA nanoparticles for GLI1 and PI3K/Akt-mediated inhibition in breast adenocarcinoma. Nanotechnology 2020;31(18):185102. https://doi.org/10.1088/1361-6528/ab6d20. PMid: 31952056.
[16] Cohen MH, Williams GA, Sridhara R, et al. United States Food and Drug Administration Drug Approval summary: Gefitinib (ZD1839; Iressa) tablets. Clin Cancer Res 2004;10(4):1212–8. https://doi.org/10.1158/1078-0432.CCR-03-0564. PMid: 14977817.
[17] Massagué J, Obenauf AC. Metastatic colonization by circulating tumour cells. Nature 2016;529(7586):298–306. https://doi.org/10.1038/nature17038. PMid: 26791720.
[18] Chen Z, Zhang M, Xu Y. Cadherin switching induced by P120-catenin can promote the migration and invasion of oral squamous cell cancer cells. West China J Stomatol 2017;35(02):183–6. https://doi.org/10.7518/hxkq.2017.02.014.
[19] Kalluri R, Weinberg RA. The basics of epithelial-mesenchymal transition. J Clin Investig 2009;119(6):1420–8. https://doi.org/10.1172/JCI39104. PMid: 19487818.
[20] Kagaya Y, Hobo T, Murata M, et al. Abscisic acid-induced transcription is mediated by phosphorylation of an abscisic acid response element binding factor, TRAB1. Plant Cell 2002;14(12):3177–89. https://doi.org/10.1105/tpc.005272. PMid: 12468735.
[21] Ashburner M, Ball CA, Blake JA, et al. Gene ontology: tool for the unification of biology. Nat Genet 2000;25(1):25–9. https://doi.org/10.1038/75556. PMid: 10802651.
[22] Meek DW. Regulation of the p53 response and its relationship to cancer. Biochem J 2015;469(3):325–46. https://doi.org/10.1042/BJ20150517. PMid: 26205489.
[23] Gao S, Li D. Research advances in tumor necrosis factor and cancer. Chinese Pharmacological Bulletin, 2020;36(09):1209-1213. [Accessed: 23 March, 2021]. Available from: http://kns.cnki.net/kcms/detail/detail.aspx?FileName=YAOL202009006&DbName=CJFQ2020.
[24] Armas-López L, Zúñiga J, Arrieta O, et al. The Hedgehog-GLI pathway in embryonic development and cancer: implications for pulmonary oncology therapy. Oncotarget, 2017;8(36):60684–703. https://doi.org/10.18632/oncotarget.19527 PMid: 28948003.
[25] Han YD, Sun YX, Zhang YS, et al. High DPP4 expression predicts poor prognosis in patients with low-grade glioma. Mol Biol Rep, 2020;47(3):2189-2196. https://doi.org/10.1007/s11033-020-05321-w PMid: 32076999.
[26] He Y, Zhong W, Li Y. Progress in the diagnosis and treatment of recurrent gliomas. Hainan Med J, 2021;32(02):246-249. [Accessed: 21 March, 2021]. Available from: http://kns.cnki.net/kcms/detail/detail.aspx?FileName=HAIN202102029&DbName=CJFQ2021.
[27] Lee EJ, Ahn KH, Hong SC, et al. Rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) chemotherapy for diffuse large B-cell lymphoma in pregnancy may be associated with preterm birth. Obstetrics Gynecol Sci 2014;57(6):526. https://doi.org/10.5468/ogs.2014.57.6.526. PMid: 25469343.
[28] Xue Q, Cao L, Chen X-Y, et al. High expression of MMP9 in glioma affects cell proliferation and is associated with patient survival rates. Oncol Lett, 2017;13(3):1325–1330. https://doi.org/10.3892/ol.2017.5567 PMid: 28454256.
[29] Zhang M, Li Y, Zhang Y. Expression and significance of MMP3, TIMP-1 and VEGF in glioma. Modern Med J China, 2014;16(02):20-22. [Accessed: 11 December, 2020].Available at: http://kns.cnki.net/kcms/detail/detail.aspx?FileName=ZHTY201402009&DbName=CJFQ2014.
[30] Oh J, Kim Y, Che L, et al. Regulation of cAMP and GSK3 signaling pathways contributes to the neuronal conversion of glioma. PLoS ONE 2017;12(11):e0178881. https://doi.org/10.1371/journal.pone.0178881. PMid: 29161257.
[31] Sokolowska P, Nowak JZ. Effects of PACAP and VIP on cAMP-generating system and proliferation of C6 glioma cells. J Mol Neurosci 2008;36(1–3):286–91. https://doi.org/10.1007/s12031-008-9071-9. PMid: 18491045.
[32] Nakagawa M, Nakashima S, Banno Y, et al. Increased expression and secretion of r-Gsp protein, rat counterpart of complement C1s precursor, during cyclic AMP-induced differentiation in rat C6 glioma cells. Mol Brain Res 2002;106(1-2):12–21. https://doi.org/10.1016/S0169-328X(02)00404-7.
[33] Van Kolen K, Slegers H. $P2Y_{12}$ receptor stimulation inhibits β-adrenergic receptor-induced differentiation by reversing the cyclic AMP-dependent inhibition of protein kinase B. J Neurochem 2004;89(2):442–53. https://doi.org/10.1111/j.1471-4159.2004.02339.x. PMid: 15056287.
[34] Walia MK, Ho PMW, Taylor S, et al. Activation of PTHrP-cAMP-CREB1signaling following p53 loss is essential for osteosarcoma initiation and maintenance. E life, 2016;5:e13446. https://doi.org/10.7554/eLife.13446 PMid: 27070462.
[35] Peverelli E, Catalano R, Giardino E, et al. Cofilin is a cAMP effector in mediating actin cytoskeleton reorganization and steroidogenesis in mouse and human adrenocortical tumor cells. Cancer Lett 2017;406:54–63. https://doi.org/10.1016/j.canlet.2017.07.025. PMid: 28826686.