Inhibition of integrin receptor by increasing concentrations and treatment durations with Cilengitide increases the sensitivity of killing in vivo of U87 glioma cancer cell xenograft mouse.

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Abstract:
This study examined the effects of integrin αvβ3 on specific cell lines and xenograft mice. The experiment used female nude mice (BALB/c mice, four weeks old). The protein is collected in a lysis buffer containing phosphatase and protease inhibitors. Cell cycle distribution was measured by FACSCalibur flow cytometry, and MTT measures the activity of cells. The results of 12 combinations were described in this study. The results of this study indicated whether Cilengitide therapy can inhibit glioma proliferation in vitro and in vivo. To conclude, the results of this study also provided some insights into future research directions in the same field. The interaction mechanism between Cilengitide and gliomas can be further investigated to provide insights into the molecular pathways and may reveal additional therapeutic targets.

Keywords: integrin αvβ3; Cilengitide; U87 glioma cancer cell; xenograft mouse.

1. Introduction:
Glioma is one of the main causes of cancer-related morbidity and mortality diseases worldwide. Five to six out of every 100,000 people in China die from glioma. Since 2017, the case fatality rate of glioma ranks second only to pancreatic cancer and lung cancer in the ranking of systemic tumors [1]. The pathogenesis of glioma is unknown, but the two risk factors identified are exposure to high doses of ionizing radiation and inherited mutations in genes with high penetrance associated with rare syndromes. Brain glioma treatment is mainly surgical resection combined with radiotherapy, chemotherapy, and other comprehensive treatment [3]. People of all ages are at risk for glioma. Different types of gliomas may occur in different sexes and different age groups. While ependymoma is more common in children and young adults, medulloblastoma mostly occurs in children [4]. It has been found that integrin αvβ3 can promote nerve repair in cerebral ischemia-reperfusion injury [5].

An integrin inhibitor, cilengitide, inhibited the αvβ3 and αvβ5 integrins and tumor progression in several preclinical assays that stimulated its testing in clinical trials [6]. Further understanding of the inhibitory molecule cilengitide in inhibiting glioma may greatly help medical treatment.

Integrins are a class of transmembrane protein receptors belonging to glycoproteins, found in monocytes, neutrophil T cells, B cells, NK cells, macrophages, dendritic cells, and platelets that mediate cell-to-cell or cell-to-extracellular matrix (ECM) interactions.

Integrin αvβ3 is a cell surface hyaluronan receptor composed of αv and β3 heterodimers. After activation, biochemical signals are transmitted into cells through downstream effector proteins, and signals can also be transmitted from the inside out. It is the most typical cell adhesion receptor. It has been shown to play an important role in various cell activities, including cell proliferation, cell differentiation, cell migration, cell survival, communication cell signal networks, gene regulation, and cytoskeletal arrangement [7]. Targeting integrin αvβ3 inhibitors can be divided into 1. chemically synthesized small molecules: (1) phenolic peptides such as the phenolic acid peptide compound cryptophycin-1 from cyanobacteria; (2) β-amino acid derivatives such as compound 33S; (3) Guanidine derivatives such as 1a-RGD peptide; 2. biological macromolecular drugs: (1) proteins such as the novel peptide ProAgio protein; (2) Conjugations of proteins and anticancer drugs such as binding protein-drug conjugations (binding agents) such as antibody-drug conjugations, polypeptide-drug conjugations, pegylated proteins (HM-3) [8]. Integrin αvβ3, a cell surface hyaluronan receptor composed of αv and β3 heterodimers, is expressed at low levels in epithelial cells of most normal tissues or organs but highly expressed in inflammatory environments, selectively upregulated in hypoxic brain tissue of the nervous system and persistently high expressed during reperfusion [9]. Continuous “cross-talk” between cells through integrins can activate intracellular signals to accelerate tumor cell proliferation, invasion, and metastasis to neighboring sites [10]. Integrins comprise two subunits, all transmembrane
proteins, except β4, which has an extracellular domain, a single transmembrane domain, and a cytoplasmic tail. Eighteen different α subunits and eight different β subunits can form 24 different αβ heterodimers between which metal ions are non-covalent bonds. Each integrin can bind to specific ligands, such as those present in ECM proteins that recognize the RGD sequence (tripeptide Arg-Gly-Asp), including laminin, fibronectin, vitellin, collagen, or other cell surface receptors of the immunoglobulin superfamily (such as intercellular adhesion molecule-1 or ICAM-1) [11].

Integrins αβ3 integrins are involved in mediating tumor cell migration, invasion, and angiogenesis, so αβ3 integrins may be an important molecule assisting in early cancer diagnosis and intervention [12]. Integrin αβ3 can degrade the extracellular matrix and weaken its barrier function by activating the expression of matrix metalloproteinase 2 (MMP-2), as well as by regulating cell adherence molecule-1 CAM-1) completes immune escape and metastasis of tumor cells [13]. The ligand binding to integrin αβ3 has a special amino acid core sequence, Arg-Gly-Asp, RGD sequence. Integrin αβ3 inhibitors designed for RGD sequence have become the targets of many clinical therapies for tumor and inflammatory diseases [14]. VEGF destroys the blood-brain barrier in the early stage of cerebral ischemia [15], and integrin αβ3 can activate VEGFR and upregulate VEGF expression [16]. Integrin αβ3 inhibitors can target the inhibition of VEGF receptor activation. Shimamura et al. found that cRGDFV treatment can reduce infarct size, alleviate brain edema, prevent fibrinogen deposition, and reduce VEGF, p-FRk-1 (VEGF receptor), and p-FAK (intracellular kinase phosphorylated in the presence of VEGF) and fibrinogen [17]. Linqing Lu found that integrin αβ3 inhibitor cilengitide could reduce brain water content, blood-brain barrier permeability, infarct size, VEGF, p-Frk, Cleaved Caspase-3 protein expression, and apoptotic cell number in MCAO (experimental rat middle cerebral artery occlusion model) [18]. Integrin αβ3 is involved in mediating tumor cell migration and invasion and angiogenesis, such as participating in the pathological process of glioblastoma.

2. Hypothesis and Controls (testable answer to the question):

"Integrin αβ3 is involved in mediating tumor cell migration and invasion and angiogenesis, such as participating in the pathological process of glioblastoma. Therefore, this study predicts that inhibition of the integrin receptor by increasing concentrations and treatment durations with Cilengitide increases the sensitivity of killing in vivo of U87 glioma cancer cell xenograft mouse. Measure tumor size by weight and volume of the xenograft mouse treated with different amounts and durations of cilengitide and vascular proliferation by FACS for CD105 and CD31."

3. Materials and Methods:

2.1 Western blotting

The protein is collected in a lysis buffer containing phosphatase and protease inhibitors. Bicinchoninic acid assay (Solarbio, Beijing) determined the protein concentration. Western blotting. The following steps are performed according to the standard scheme. Antibodies against integrin αβ3, e-cadherin, and n-cadherin must be obtained from the company.

2.2 Xenograft mouse

The experiment used female nude mice (BALB/c mice, four weeks old). About 2 × 10^6 cells transfected with U87 cells were injected subcutaneously on both sides of BALB/c mice. Tumor size was measured every 3-4 days. After 30 days, the animals were killed, and the tumors weighed.

2.3 FACTS

Cell cycle distribution was measured by FACSCalibur flow cytometry (Becton Dickinson, San Jose, CA). About 1 × 10^6 cells were harvested and fixed with 75% ethanol for 4 hours. The cells were washed with PBS and stained in the dark with propyl iodide working solution for 30 minutes. The stained cells were then analyzed by flow cytometry. In each experiment, 20,000 events were recorded for each sample. The cell data of the G0/G1, S, and G2/M stages are presented in Table 1.

2.4 MTT

MTT measures the activity of cells. Dissolve 5mg /ml MTT solution in PBS, filter, and disinfect. 5 hours before the end of incubation, 20µl of the MTT solution from
the first step was added to each well. Culture in a CO2 incubator at 37°C for 5 hours. Remove the media with a needle and syringe. Add 200 DMSO to each well and dissolve the crystals with the up-down pipette. Place the plate in an incubator at 37°C for 5 minutes. Transfer to a tablet reader and measure the absorbance at 550nm.

3 Possible Results

<table>
<thead>
<tr>
<th>Combination of possible results (CR)</th>
<th>Increase in vitro killing (MTT)</th>
<th>E-cadherin and n-cadherin (western blotting)</th>
<th>Cell cycle (FACS)</th>
<th>Decrease in xenograft tumor size (xenograft mouse)</th>
<th>Supporting hypothesis?</th>
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<tbody>
<tr>
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For e-cadherin and n-cadherin, “+” represents the results of the increase in e-cadherin and the decrease in n-cadherin, “-” represents the results of the decrease in e-cadherin and the increase in n-cadherin. For the cell cycle, “+” represents that the cells at the G0/G1 phase increase and the cells at the G2/M phase decrease, and “-” represents that the cells at the G0/G1 phase decrease and the cells at the G2/M phase increase.

3.1 Description of each combination

Combination of possible results 1 (CR1): Compared with the control group, the use of Cilengitide showed a significant decrease in the number of glioma cells in the belly of the mice after the xenograft assay. The western blotting analysis also showed a thicker band for integrin αvβ3. Moreover, the glioma tumor’s growth rate was lower in the Cilengitide treated mice than the controlled mice. Additionally, the size of the tumor of the Cilengitide-treated mice also decreased.

Combination of possible results 2 (CR2): Compared with the control group, the use of Cilengitide showed a significant decrease in the number of glioma cells in the belly of the mice after the xenograft assay. The western blotting analysis also showed a thicker band for integrin αvβ3. Moreover, the glioma tumor’s growth rate was lower in the Cilengitide treated mice than the controlled mice. However, compared with the control group, the tumor size was found to be similar or bigger in the Cilengitide-treated mice.

Combination of possible results 3 (CR3): Compared with the control group, the use of Cilengitide showed a significant decrease in the number of glioma cells in the belly of the mice after the xenograft assay. The western blotting analysis also showed a thicker band for integrin αvβ3. Moreover, the glioma tumor’s growth rate was lower in the Cilengitide treated mice than the controlled mice. However, the rate of growth of the glioma tumor was found to be similar or higher in the Cilengitide-treated mice compared to the controlled mice. Additionally, the size of the tumor of the Cilengitide-treated mice decreased.

Combination of possible results 4 (CR4): Compared with the control group, the use of Cilengitide showed a
significant decrease in the number of glioma cells in the belly of the mice after the xenograft assay. However, the western blotting analysis showed a similar or thinner band for integrin αvβ3. Moreover, the glioma tumor’s growth rate was lower in the Cilengitide-treated mice than the controlled mice. Additionally, the size of the tumor of the Cilengitide-treated mice also decreased.

Combination of possible results 5 (CR5): The use of Cilengitide showed no significant decrease in the number of glioma cells; however, a similar or lower survival rate in the MTT assay compared to the control group. The western blotting analysis showed a thicker band for integrin αvβ3. Moreover, the glioma tumor’s growth rate was lower in the Cilengitide-treated mice than the controlled mice. Additionally, the size of the tumor of the Cilengitide-treated mice also decreased.

Combination of possible results 6 (CR6): Compared with the control group, the use of Cilengitide showed a significant decrease in the number of glioma cells in the belly of the mice after the xenograft assay. The western blotting analysis also showed a thicker band for integrin αvβ3. However, the glioma tumor’s growth rate was similar in the Cilengitide-treated mice compared to the controlled mice. Additionally, the tumor size was found to be similar in the Cilengitide-treated mice.

Combination of possible results 7 (CR7): Compared with the control group, the use of Cilengitide showed a significant decrease in the number of glioma cells in the belly of the mice after the xenograft assay. Moreover, the glioma tumor’s growth rate was lower in the Cilengitide-treated mice than the controlled mice. However, the western blotting analysis showed a similar band for integrin αvβ3. Additionally, the tumor size was found to be similar in the Cilengitide-treated mice.

Combination of possible results 8 (CR8): Compared with the control group, the use of Cilengitide showed a significant decrease in the number of glioma cells in the belly of the mice after the xenograft assay. Additionally, the size of the tumor of the Cilengitide-treated mice also decreased. However, the western blotting analysis showed a similar band for integrin αvβ3. Moreover, the glioma tumor’s growth rate was similar or higher in the Cilengitide-treated mice compared to the controlled mice.

Combination of possible results 9 (CR9): The use of Cilengitide showed no significant decrease in the number of glioma cells; however, a similar survival rate in the MTT assay compared to the control group. Moreover, the western blotting analysis showed a thicker band for integrin αvβ3. Additionally, the size of the tumor of the Cilengitide-treated mice was found to be higher in the Cilengitide-treated mice compared to the controlled mice.

Combination of possible results 10 (CR10): The use of Cilengitide showed no significant decrease in the number of glioma cells; however, a similar survival rate in the MTT assay compared to the control group. The western blotting analysis also showed a similar band for integrin αvβ3. Moreover, the glioma tumor’s growth rate was lower in the Cilengitide-treated mice than the controlled mice. Additionally, the size of the tumor of the Cilengitide-treated mice also decreased.

Combination of possible results 11 (CR11): The use of Cilengitide showed no significant decrease in the number of glioma cells; however, a similar survival rate in the MTT assay compared to the control group. Additionally, the tumor size was found to be similar in the Cilengitide-treated mice. The western blotting analysis showed a thicker band for integrin αvβ3. Moreover, the glioma tumor’s growth rate was lower in the Cilengitide-treated mice than the controlled mice.

Combination of possible results 12 (CR12): Compared with the control group, the use of Cilengitide showed a significant decrease in the number of glioma cells in the belly of the mice after the xenograft assay. However, the western blotting analysis showed a thinner band for integrin αvβ3. Moreover, the rate of growth of the glioma tumor was found to be higher in the Cilengitide-treated mice compared to the controlled mice. Additionally, the tumor size was found to be bigger in the Cilengitide-treated mice.

Combination of possible results 13 (CR13): The western blotting analysis showed a thicker band for integrin αvβ3. Cilengitide showed no significant decrease in the number of glioma cells; however, there was a lower survival rate in the MTT assay compared to the control group. Moreover, the rate of growth of the glioma tumor was found to be higher in the Cilengitide-treated mice compared to the controlled mice. Additionally, the tumor size was found to be bigger in the Cilengitide-treated mice.

Combination of possible results 14 (CR14): The glioma tumor’s growth rate was lower in the Cilengitide-treated mice than the controlled mice. Cilengitide showed no significant decrease in the number of glioma cells; however, there was a lower survival rate in the MTT assay compared to the control group. The western blotting analysis also showed a thinner band for integrin αvβ3. Additionally, the tumor size was found to be bigger in the Cilengitide-treated mice.

Combination of possible results 15 (CR15): The size of the tumor of the Cilengitide-treated mice decreases. Cilengitide showed no significant decrease in the number of glioma cells; however, there was a lower survival rate in the MTT assay compared to the control group. The
western blotting analysis also showed a thinner band for integrin αvβ3. Moreover, the rate of growth of the glioma tumor was found to be higher in the Cilengitide treated mice compared to the controlled mice.

Combination of possible results 16 (CR16): The use of Cilengitide showed no significant decrease in the number of glioma cells; however, a lower survival rate in the MTT assay compared to the control group. The western blotting analysis also showed a thinner band for integrin αvβ3. The western blotting analysis also showed a thinner band for integrin αvβ3. Moreover, the rate of growth of the glioma tumor was found to be higher in the Cilengitide treated mice compared to the controlled mice. Additionally, the tumor size was found to be bigger in the Cilengitide-treated mice.

4 Discussion

Since the 1980s, many roles of integrins, a family of adhesion molecules, have been documented, which have increased the understanding of neural development, synaptic function, and several neurological diseases. Among so many experiments, the role of integrins in the neural crest was mainly verified [19]. The neural crest is a population of migrating cells from the dorsal neural tube, distributed in the sensory, autonomic, and enteric nervous systems and contributes to the formation of many specialized sensory organs, glands, the heart, cranial mesenchyme, and bone, as well as supporting cells in the peripheral nerves, including chevron cells and endoneurial fibroblasts [20]. Neural crest cells express many integrins and migrate through the ECM-rich environment [21]. The effect of integrins on the neural crest is mainly growth inhibition. For example, acute inhibition experiments in bird embryos demonstrated the important role of integrins in neural crest migration [22]. In mice, genetic ablation of β1 integrins leads to severe perturbations in the peripheral nervous system, including failure of normal neural dendrites, delayed migration of Schwann cells, and defects in neuromuscular junction differentiation [23]. In addition to the direct effects on migration, it has been shown that the lack of specific integrin heterodimers impairs the survival, proliferation, and differentiation of snowwang cell precursors. These observations may reflect the role of integrin receptors in regulating the activation of MAP kinase, Rac, and other signaling pathways. Based on the effects of integrins in the nervous system, this study hypothesized that integrin αvβ3 might positively affect tumor suppression. Therefore, this research proposal focuses on the effect of Cilengitide formed from integrin αvβ3 on gliomas.

In combination with possible result 1 (CR1), MTT experiments showed higher survival rates in the Cilengitide-treated group, meaning that fewer cancer cells survived the experiments and suggesting that the therapy may also hinder cancer growth in the body. In addition, the results of the Western blot showed elevated expression in the Cilengitide-treated group, suggesting that Cilengitide may be able to cause the death of cancer cells. The number of cells was also reduced in the Cilengitide-treated group compared to the control group, suggesting that integrin αvβ3 may reduce the growth of glioma cells. Finally, the inhibitory effect of integrin αvβ3 on gliomas is supported by the fact that gliomas in the Cilengitide-treated group grew slower than the control group in animal experiments. Overall, these CR1 results provide strong evidence in support of the study hypothesis.

In CR2, MTT, western blot, and FACS experiments showed that integrin αvβ3 had a complete inhibitory effect on cancer cell growth in vitro. The experimental results of all three experiments were positive; however, no in vivo effect of the treatment was observed in this study. There are two possible explanations for this result: either this combination did not have any in vivo effect on gliomas, or the dose of Cilengitide given to the mice was too low to fully exploit its anticancer properties. To investigate this further, it is recommended that the animal experiments be repeated with higher doses. Similarly, in CR7 and CR11, the therapy did not show any antiproliferative effect on cancer cells in vivo, suggesting that Cilengitide therapy may only affect glioma proliferation on a small scale.

In CR3, it is noteworthy that Cilengitide therapy did not inhibit glioma growth in vitro but did inhibit tumor growth in vivo. This pattern can also be observed in several other results combinations, such as CR8 and CR9. These effects, not observed in vitro but observed in vivo, are unexpected and contradict the hypothesis. One possible explanation for this result is that there may have been an error during the animal experiments, e.g., the addition of unknown agents capable of inhibiting the tumor. Another possibility is that the number and quality of tumor cells were not standardized, leading to differences in the MTT assay. However, in this case, the experiment should be repeated with the same experiment to ensure its accuracy.

In CR4 and CR5, Cilengitide treatment inhibited glioma cell growth in vitro and in vivo. Still, e-cadherin and n-cadherin release, as well as cellular activity, were not observed. We used western blot to investigate the reversal of Cilengitide in cancer cells. Although the results of western blot or MTT assays contradicted the hypothesis, other results of CR4 and CR5 supported the hypothesis, suggesting that apoptosis in cancer cells may occur through a different mechanism than experimentally expected. These combinations only partially support
the hypothesis. However, in CR12 and CR13, treatment did not inhibit tumor growth, which contradicts the hypothesis. Such results may suggest that integrin αvβ3 is induced rather than targeted. For the possibility of these results, errors may occur in the MTT assay and Western blot.

In CR6, MTT and Western blot assays showed that Cilengitide therapy was inductive but did not affect cancer cells. The findings suggest two possible reasons for this. One is due to errors or mistakes in the western blot and MTT testing process, which led to the non-conformity of the experimental results. More careful experiments should be repeated with extra care. Another reason is that Cilengitide therapy causes other cells to increase or react, an unintended consequence that may bring extreme side effects. In this case, this study should change the test subject or review the information to determine the safety of Cilengitide therapy.

In CR10, CR14, and CR15, Cilengitide therapy did not show a response to gliomas but inhibited cancer cell growth in vitro or in vivo, or both. However, since this experiment did not investigate where cancer cells were inhibited, it is too early to conclude Cilengitide therapy. Because CR10, CR14, and CR15 are inconclusive about the cancer inhibition ability of Cilengitide therapy and can only partially support this hypothesis. This study should set up more possibilities and repeat the experiments to determine the agents that work for medical treatment in its process.

CR16 was the most unexpected result of all the combinations because it showed that each experiment contradicted the hypothesis. This suggests that integrin αvβ3 may have no role in treating gliomas even though it has shown anticancer effects in other systems.

5 Conclusion

Overall, this study examined the effects of integrin αvβ3 on specific cell lines and xenograft mice, i.e., the degree of inhibition of gliomas. The results of this study indicate whether Cilengitide therapy can inhibit glioma proliferation in vitro and in vivo. Thus, the promising therapeutic effects of Cilengitide therapy will set the stage for its transition to clinical trials. The results of this study will also provide some insights into future research directions in the same field. The interaction mechanism between Cilengitide and gliomas can be further investigated to provide insights into the molecular pathways involved and may reveal additional therapeutic targets in the process. Cilengitide can also be used with other glioma anticancer drugs (e.g., Temozolomide). The cancer inhibitory effect may be more pronounced. Potential synergistic effects between the drugs could also be examined.

References

Mechanism of action of Cilengitide, integrin αvβ3 inhibitor, in acute cerebral ischemia in rats [J], Chinese Medical Journal, 96(7):559-564.


