Valproic Acid Enhanced Apoptosis by Promoting Autophagy Via Akt/mTOR Signaling in Glioma

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Abstract
Glioma is the most common malignant tumor in the central nervous system with a poor median survival. Valproic acid (VPA), a widely used antiepileptic drug, has been found to have antitumor effects on gliomas, but its role still has not been determined. In this study, we investigated VPA-induced apoptotic and autophagic effects on human U251 and SNB19 cells by cell counting kit-8 assay, flow cytometry, terminal deoxynucleotidyl transferase-mediated nick end labeling staining, western blots, and immunofluorescence assay in vitro, and then we further explored the role of autophagy in apoptosis by using the selective antagonist MHY1485. The data showed that VPA inhibited U251 and SNB19 glioma cells viability in a dose-dependent and time-dependent manner and induced apoptosis through the mitochondria-dependent pathway in vitro. In addition, VPA activated the Akt/mTOR pathway by decreasing their protein phosphorylation to promote cellular apoptosis. Surprisingly, the mTOR agonist MHY1485, causing a strong elevation of mTOR activity, partially reduced apoptosis ratio, which supposing that the autophagy of VPA is involved in the regulation of apoptosis. These findings suggest that VPA enhanced apoptosis by promoting autophagy via Akt/mTOR signaling in glioma, which could be further evaluated as a reliable therapy for glioma.

Keywords
valproic acid, apoptosis, autophagy, Akt/mTOR signaling, glioma

Introduction
Glioma, which is the most prevalent and aggressive primary intracranial tumor, accounts for almost 80% of brain tumors.¹,² Conventional therapies for glioma include surgical resection, chemotherapy, and radiotherapy.³ Temozolomide (TMZ), a new alkylating antineoplastic agent, has become the first-line glioma chemotherapy drug. TMZ contributes to cell cycle arrest³ and apoptosis⁵ via alkylation damage of tumor cell DNA⁴. However, TMZ is not sensitive or effective in gliomas with high O⁶-methylguanine-DNA methyltransferase (MGMT) expression levels and unmethylated promoter regions.² In addition, cellular DNA base excision repair, mismatch repair, nucleotide excision repair, and homologous recombination are involved in glioma resistance to TMZ⁶. These facts demonstrate that the comprehensive treatment effects of TMZ on glioma, including high invasiveness and recurrence rates along with a median survival of 14.6 months, are far from satisfactory⁹. Therefore, it is crucial to investigate promising and reliable drugs for glioma therapy.

Valproic acid (VPA), a monobranched carboxylic acid, is a clinical antiepileptic drug. As one of the most common histone deacetylase inhibitors (HDACIs), VPA has been found to have antitumor effects on a variety of solid tumors, including glioma.¹⁰ In multiple glioma models, VPA exerted many different antitumor effects, including antiproliferation, anti-invasion, antiangiogenesis, and proapoptosis activities.¹¹ According to cell proliferation assays, VPA inhibited glioma cell viability by Wnt/β-catenin signaling activation via inducing the transcriptional activity of the β-catenin/TCF
complex\textsuperscript{12}, and it impaired glioma cell invasion in vitro through decreases in matrix metalloproteinases MMP2 and MMP9 and an increase in the inhibitor tissue inhibitor MMP1\textsuperscript{13}. Regarding its antiangiogenesis effects, VPA reduced vascular endothelial growth factor secretion in glioma cells in a dose-dependent manner and suppressed tube formation and endothelial cell proliferation by targeting TE-1\textsuperscript{14}. In addition, VPA promoted glioma cell apoptosis by targeting the PON2-Bim cascade\textsuperscript{15}, the RECK-MMP pathway, mitogen-activated protein kinase pathways,\textsuperscript{16} and nuclear factor kappa B activation\textsuperscript{17}. Autophagy is the natural and orderly process to eradicate cytoplasmic organelles or unused proteins and maintain cellular homeostasis\textsuperscript{18}, which plays both positive and negative roles in cellular activities and tumor formation\textsuperscript{19}. It provides energy for cell functions through the degradation of molecules and cellular organs, but it also reduces cell damage by promoting the elimination of pathogens, toxic molecules, damaged cellular organelles, and misfolded proteins\textsuperscript{20,21}. However, excess autophagy can induce autophagic cell death via the excessive degradation of mitochondria and damaged molecules required for cell survival\textsuperscript{22}. Therefore, autophagy, an important and conservative catabolic process in the evolutionary process, is becoming a key player in tumor resistance research\textsuperscript{23}. VPA can promote autophagy in glioma cells\textsuperscript{24–26}, and follow-up studies have focused mainly on the effect of VPA as an adjuvant-sensitizing chemotherapy drug\textsuperscript{27,28}. Similarly, Laurence revealed that VPA could induce apoptosis and autophagy of glioblastomas via increased cleaved caspase-3 and reduced LC3-II, p62, and p-mTOR\textsuperscript{29}. However, autophagy induction by VPA alone and the mechanism in glioma cells remain unclear. More importantly, the interaction between apoptosis and autophagy mediated by VPA is still uncertain.

In the present study, we examined the role of VPA in the promotion of apoptosis and autophagy in gliomas. Furthermore, we screened the underlying mechanism through which VPA promoted autophagy and investigated the interaction between apoptosis and autophagy. Taken together, our data showed that the application of VPA may provide a more reliable therapeutic strategy for treating glioma.

**Materials and Methods**

**Cell Culture and Materials**

Two glioma cell lines, U251 and SNB19, were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and incubated in 5% carbon dioxide at 37 °C. The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 U/mL penicillin and 100 mg/L streptomycin).

VPA and MHY1485 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide was purchased from MP Biomedicals LLC (Santa Ana, CA, USA). Antibodies, including β-actin, caspase-3, cleaved caspase-3, Bcl-2, Bax, p62, LC3B, AKT, p-AKT, mTOR, and p-mTOR, were purchased from Cell Signaling Technology (Danvers, MA, USA). The secondary biotinylated antibodies were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA).

**Cell Viability Assay**

Cells were seeded into a 96-well plate at 2 × 103 cells per well and incubated overnight at 37 °C. The cells were then incubated with VPA dissolved in sterile water at concentrations ranging from 0 to 4.0 mM for 24 h, 48 h, and 72 h. Subsequently, 10 μL of cell counting kit-8 (CCK-8) solution (Bimake, Houston, TX, USA) was added to each well, and the plate was incubated for 2 to 4 h at 37 °C on a shaker. The absorbance value was measured at 490 nm on an EL×800 (BioTek, Winooski, VT, USA). The viability of untreated cells was regarded as 100%, and the half-maximal inhibitory concentration (IC\textsubscript{50}) was calculated by Prism 5.0. The experiment was repeated three times.

**TUNEL Assay**

Initially, cells were cultured in a 96-well plate with 1 × 103 cells per well and incubated overnight at 37 °C. The cells were then incubated with VPA at concentrations of 0 and 2 mM in combination with MHY1485 (1 μM) for 48 h. The supernatant was removed, and the cells were washed with phosphate-buffered saline (PBS) three times. A terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay (TUNEL kit, Roche, Basel, Switzerland) was employed to detect cell apoptosis. Cells were fixed with 4% paraformaldehyde at room temperature for 25 min and washed with 0.2% Triton X-100 in PBS three times. All procedures for the TUNEL assay followed the standard protocol. After fluorescent staining, we used a fluorescence microscope (Nikon, Kawasaki, Japan) to review the slides and quantify the extent of apoptosis. Finally, we examined representative images after 48 h of VPA treatment.

**Cell Apoptosis Assay**

Cells were seeded into 6-well plates and grown to >80% confluence. The cells were then treated with VPA, MHY1485, or VPA + MHY1485 at the indicated concentrations for 48 h. Next, the cells were collected and washed completely with PBS. After centrifugation, 1 × 105 cells were resuspended in binding buffer (1×) and stained with 5 μL Annexin V-fluorescein isothiocyanate and 5 μL propidium iodide (BD Biosciences Pharmingen, San Diego, CA, USA) for 15 to 30 min. Immediately, the cells were detected by flow cytometry (Guava EasyCyte 6HT-2 L, Merck Millipore, Darmstadt, Germany).

**Western Blotting**

Cells were cultured in a 6-well plate and treated with VPA, MHY1485, or both at the indicated concentrations for 48 h.
The cells were collected, washed with PBS three times, and then lysed in radio immunoprecipitation assay buffer (Beyotime, Shanghai, China) containing a 1% protease and phosphatase inhibitor cocktail (Roche). The supernatant was collected after centrifugation at 12,000 rcf for 2 min at 4°C. A standard bicinchoninic acid Protein Assay Kit (Thermo Fisher Scientific, MA, USA) was applied to determine the protein concentration. Western blot analysis was performed according to a standard protocol. Primary antibodies against caspase-3 (#9662), cleaved caspase-3 (#9664), Bax (#2772), Bcl-2 (#2872), p62 (#8025), LC3 (#3868), mTOR (#2983), p-mTOR (#5536), AKT (#4691), p-AKT (#4060), and β-actin (#4970) were purchased from Cell Signaling Technology. Chemiluminescent signals were detected with an ECL plus kit (Thermo Fisher Scientific) on a ChemiDoc Touch Imaging System (BioRad, CA, USA).

**Immunofluorescence Assay**

Cells were cultured in a 24-well plate and transfected with adeno virus expressing mCherry-GFP-LC3-II plasmid for 24h. Then the cells were treated with VPA, MHY1485, or both at the indicated concentrations for another 48 h. The confocal microscopy was utilized to visualize the images of the cells and capture the representative pictures. The Autophagic flux was measured by the color change of GFP-mCherry.

**Statistical Analysis**

The data are expressed as the mean ± SD. Statistical analysis was performed using Student’s t-test (2-tailed) or one-way analysis of variance (Bonferroni test). The criterion for statistical significance was set as \( P < 0.05 \).

**Results**

**VPA Inhibited Glioma Cell Viability**

To determine the antitumor effects of VPA on glioma cells, the common glioma cell lines U251 and SNB19 were selected for subsequent experiments, of which the cellular morphologies are shown in Fig. 1A. The viability of the two glioma cell lines was detected by CCK-8 assay under incubation with VPA at the concentration ranging from 0 to 4 mM for 0, 24, 48, 72 h. As shown in Fig. 1B, VPA inhibited the proliferation of the two glioma cell lines in a dose-dependent and time-dependent manner. Fig. 1B also illustrated that the optimal concentration and incubation time for U251 cells were 2 mM and 48 h, while the optimal concentration and incubation time for SNB19 cells were 2 mM and 24 h. Moreover, the IC50 values of VPA on U251 cells at 24, 48, and 72 h were 8.52, 7.45, and 6.05 mM, respectively. The IC50 values of VPA on SNB19 cells at 24, 48, and 72 h were 8.61, 7.32, and 6.20 mM, respectively (Fig. 1B). Considering the toxicity of VPA on normal and glioma cells, the concentration of 2 mM and the incubation time of 48 h were applied for the follow-up experiments consequently.

**VPA Promoted Glioma Cell Apoptosis**

To evaluate apoptosis induction by VPA, TUNEL assay and flow cytometry were carried out. In Fig. 2A, normal cells were dyed blue (4’,6-diamidino-2-phenylindole) and apoptotic cells were dyed green (TUNEL). Compared with control conditions, treatment with VPA induced a higher apoptosis rate in both U251 and SNB19 cells. In addition, Fig. 2B demonstrates that the apoptosis rate was 12.25% for U251 cells treated with 2 mM VPA for 48 h compared with 6.10% for control U251 cells, while the apoptosis rate was 13.80% for SNB19 cells treated with 2 mM VPA for 48 h compared with 6.33% for control SNB19 cells. The statistical analysis of the apoptosis rates in U251 and SNB19 cells is shown in Fig. 2C.

To explore the potential molecular mechanism of glioma cell apoptosis initiation by VPA in U251 and SNB19 cells, some key apoptosis-associated proteins were examined by western blotting. As shown in Fig. 2D, while there was no evident fluctuation in total caspase-3 levels, cleaved caspase-3 levels moderately increased when U251 and SNB19 cells were treated with 2 mM VPA for 48 h. Moreover, Fig. 2E showed obvious accumulation of Bax and Bak and reduction of Bcl-2, Bcl-xl, and Mcl-1 in VPA-treated
U251 and SNB19 cells. The statistical analysis of cleaved caspase-3/caspase-3, Bak, Bcl-xl, Mcl-1, and Bax/Bcl-2 in U251 and SNB19 cells is shown in Fig. 2E. Taken together, VPA promoted U251 and SNB19 cell apoptosis by activating the mitochondria-dependent pathway.

VPA Promoted Glioma Cell Autophagy Via Akt/mTOR Pathway

To measure the effect of VPA on autophagy in U251 and SNB19 cells, western blot analysis was performed. As shown in Fig. 3A and B, p62 expression was downregulated, while LC3-II expression was obviously upregulated. Next, the protein expression levels of the key regulators of Akt/mTOR signaling in cellular autophagy (Akt, p-Akt, mTOR, and p-mTOR) were detected. As shown in Fig. 3C and D, p-mTOR and p-Akt expression was downregulated, but there was no significant difference between mTOR and Akt. The statistical analysis of p-mTOR/mTOR and p-Akt/Akt in U251 and SNB19 cells is shown in Fig. 3C and D. In summary, we hypothesized that VPA promoted autophagy in glioma cells, which may be related to Akt/mTOR pathway inhibition.

VPA Inhibited the Akt/mTOR Pathway

To further verify our hypothesis and clarify the underlying mechanisms of the effect of VPA on cellular autophagy, we used MHY1485, which is known to act as an mTOR agonist, to inhibit autophagy. The immunofluorescence results showed that VPA induced cell autophagy, which could be partially reduced by MHY1485, through evaluation of autophagosomes and autolysosomes in U251 and SNB19 cells (Fig. 4A–B). We found that in the VPA group, p62 expression decreased, and LC3-II expression increased, which again confirmed the autophagic effect of VPA (Fig. 4C and D). Meanwhile, p62 expression was upregulated, and LC3-II expression was downregulated upon MHY1485 treatment alone (Fig. 4C and D). These results indicated that MHY1485 could inhibit autophagy in glioma cells. Subsequently, VPA and MHY1485 were combined, and we found that compared with MHY1485 treatment alone, this combination upregulated p62 expression and downregulated LC3-II expression (Fig. 4C and D), suggesting that VPA-induced autophagy was inhibited. Further detection of mTOR and Akt expression showed that the p-mTOR/mTOR ratio was increased in the combination group, but this ratio was decreased compared with that in the VPA group (Fig. 4E).
This result suggested that MHY1485, an mTOR activator, could activate p-mTOR and thus inhibit p-Akt expression. In conclusion, MHY1485 inhibited VPA-induced autophagy by activating the Akt/mTOR pathway in U251 and SNB19 cells.

VPA Enhanced Apoptosis by Promoting Autophagy Appropriately

To investigate the interaction between apoptosis and autophagy initiated by VPA, cells were treated with MHY1485 and subjected to TUNEL assays and flow cytometry. As shown in Fig. 5A–C, MHY1485 had no definite toxic effects on either glioma cell line, which indicated that it could be used for further investigation. Similarly, VPA induced apoptosis in the two glioma cells with or without MHY1485 treatment. Intriguingly, the autophagy inhibitor MHY1485 distinctly reduced VPA-induced apoptosis in the two glioma cell lines (Fig. 5A–C).

To verify the influence of MHY1485 on apoptosis in the two glioma cell lines at the protein level, some key proteins, including caspase-3, Bax, Bak, Bcl-xl, Mcl-1, and Bcl-2, were detected. As shown in Fig. 5D, the cleaved caspase-3/caspase-3, Bak, Bcl-xl, Mcl-1, and Bax/Bcl-2 expression levels were not different between the MHY1485 group and the control group for both glioma cell lines. Significantly, VPA led to the gradual accumulation of cleaved caspase-3, Bax and Bak, and Bcl-2, Bcl-xl, and Mcl-1 expression decreased moderately, which was partially weakened by MHY1485 (Fig. 5E). In summary, VPA accelerated autophagy and apoptosis in glioma cells. However, when mTOR was activated and autophagy was inhibited, VPA-induced cell apoptosis decreased as well. Therefore, we hypothesized that VPA-induced autophagy is involved in the regulation of apoptosis, which is related to mTOR inhibition.

Discussion

Glioma, derived from the neuroepithelium, is the most common primary brain tumor in adults. It is an aggressive, invasive, and destructive malignancy that resists multiple therapies, including surgery, radiation, conventional chemotherapy, and other adjuvant therapies. TMZ is the most commonly used chemotherapy drug and is effective initially, but the subsequent resistance has become the biggest obstacle in current glioma treatment. Therefore, repurposing existing drugs has become a potential treatment option, which led to the application of VPA in our experiment. As the most frequently used antiepileptic drug (AED) in neurosurgery, VPA has been reported to have inhibitory effects on the progression, invasion, and apoptosis of glioma cells. However, the underlying molecular mechanism has not yet been clarified, making it difficult to obtain satisfactory results in limited clinical trials. In our study, we validated the enhanced apoptosis and autophagy induced by...
VPA in glioma cells and studied the interaction between these processes.

VPA is an AED with similar properties as HDACIs, which play key roles in regulating epigenetic gene expression and have been used increasingly as anticancer agents. In vitro, VPA exerted various pharmacological effects on several aspects of glioma, including cellular proliferation, invasion, cell cycle progression, and apoptosis. Notably, a study by Greg et al revealed that patients treated with VPA had significantly longer survival than those who did not receive an AED. This has resulted in attention to the anticancer potential of VPA. Consistent with these studies, our study confirmed that VPA inhibited cell viability and facilitated cellular apoptosis in glioma cells in vitro. Moreover, based on the study by Laurence et al, we further detected the expression level of Bax, Bak, Bcl-2, Bcl-xl, and Mcl-1, which play vital roles in mitochondrial apoptosis and cell death. While dying cells often manifest large-scale

**Figure 4.** Autophagy inhibitor (MHY1485) inhibited cell autophagy in glioma cells induced by VPA by activating AKT/mTOR signaling. (A) Representative images of the immunofluorescence assay of U251 and SNB19 cells after treatment with VPA (2 mM), MHY1485 (1 μM), or VPA (2 mM) + MHY1485 (1 μM) for 48 h. (B) Statistical analysis of autophagosomes and autolysosomes in U251 and SNB19 cells from immunofluorescence results. (C) The protein levels of p62 and LC3B in U251 cells, exposed to VPA (2 mM), MHY1485 (1 μM), or VPA (2 mM) + MHY1485 (1 μM) for 48 h, were evaluated by western blot analysis. Statistical analysis of p62 and LC3B protein level in U251 cells from western blot results. (D) The protein levels of p62 and LC3B in SNB19 cells, exposed to VPA (2 mM), MHY1485 (1 μM), or VPA (2 mM) + MHY1485 (1 μM) for 48 h, were evaluated by western blot analysis. Statistical analysis of p62 and LC3B protein levels in SNB19 cells from western blot results. (E) The protein levels of p-mTOR, mTOR, p-AKT, and AKT in U251 cells, exposed to VPA (2 mM), MHY1485 (1 μM), or VPA (2 mM) + MHY1485 (1 μM) for 48 h, were evaluated by western blot analysis. Statistical analysis of p-mTOR/mTOR and p-AKT/AKT in U251 cells from western blot results. (F) The protein levels of p-mTOR, mTOR, p-AKT, and AKT in SNB19 cells, exposed to VPA (2 mM), MHY1485 (1 μM), or VPA (2 mM) + MHY1485 (1 μM) for 48 h, were evaluated by western blot analysis. Statistical analysis of p-mTOR/mTOR and p-AKT/AKT in SNB19 cells from western blot results. *P < 0.05, **P < 0.01, ***P < 0.001. VPA: valproic acid.
Autophagosome accumulation in tumor cells, we hold the opinion that, to a large extent, autophagy is rarely, if ever, the mechanism by which cells actually die. Indeed, autophagy may maintain optimal, high ATP levels to facilitate the apoptotic process and initiate cell death catabolism, thus accelerating cell death.

Autophagy is a highly conserved process that is essential for cell survival, host defense, and energy consumption. Autophagy in cancer has often been described as a “double-edged sword”, which either promotes tumor survival under microenvironmental stress and increases growth and aggressiveness or suppresses tumorigenesis via its quality control function. Until now, the autophagy-promotion effects of VPA have been detected via the upregulation of LC3-I conversion to LC3-II, which correlates with the number of autophagosomes. However, p62, a selective autophagy adaptor/receptor, binds ubiquitinated proteins and LC3 for engulfment, and results for this factor have not been reported. In our study, differing from the study by Lawrence et al., we demonstrated that a visible decrease in p62 and an increase in LC3-II were detected in glioma cells incubated with VPA. To the best of our knowledge, multiple metabolic signaling pathways, including ROS, ERK, PI3K/Akt/mTOR, AMPK, and p53, have also been explored for their potential effect on autophagy. In glioma cells, a study by Fu Jun et al. suggested that the ERK pathway is involved in VPA-induced autophagy by increasing ERK 1/2 phosphorylation in glioma cells. Surprisingly, we discovered decreased expression levels of p-Akt/Akt and p-mTOR/mTOR in glioma cells after incubation with VPA, implicating Akt/mTOR signaling inhibition.

Apoptosis and autophagy are two distinct self-destructive processes determining cell fate under physiological and pathological conditions. To the best of our knowledge, there are three broadly defined categories of cell death processes: apoptosis (type I), autophagic cell death (type II), and necrosis (type III). Notably, a phrase called autophagic apoptosis summarizes the interactions between apoptosis and autophagy. In autophagic apoptosis, specific autophagy proteins can regulate apoptosis via mechanisms that are not related to their canonical role in autophagic signaling. A study by Wang Feifan et al. illustrated that hydroxycamptothecin induced apoptosis in human bladder cancer cells through AMPK-mTOR-ULK1 axis activation-dependent autophagy. Similar to our study, the reduction in autophagy by MHY1485, an autophagy inhibitor targeting mTOR, markedly alleviated the apoptosis partially promoted by VPA in glioma cells. Consistent with this, our study
illustrated that p-mTOR/mTOR and p62 upregulation and LC3-II downregulation were accompanied by decreases in cleaved caspase3/caspase3 and Bax/Bcl-2. Mechanistically, MHY1485 simultaneously triggered a decrease in p-Akt.

Meanwhile, it has been reported that VPA markedly inhibited the proliferation of glioma cells through increase of glycosphingolipid (GM3) level and reduced phosphorylation of estimated glomerular filtration rate. VPA was also confirmed to exert antitumor effects, including cell viability, proliferation, and clonogenicity on glioma. Whereas, a study conducted by Kratzsch et al revealed that VPA did not show any growth reduction epigenetically. Therefore, our results have become assertive evidence for the suppressive role of VPA in glioma. Mechanically, several investigations illustrated that activation of Wnt/β-catenin signaling was found in the activities of VPA. Moreover, VPA was verified to be involved in ERK/Akt and Ca²⁺ signaling. Our study further testified the role of Akt/mTOR signaling, which may provide a novel strategy for glioma treatment. Further in vivo experiments found that VPA prevented invasion in surrounding tissues and inhibited tumor angiogenesis. While Chen et al reported that amphiregulin (AR) secretion induced by VPA conferred resistance to TMZ. Clinically, only a few phase I trials have unfolded that VPA was probably correlated with improved survival, histological progression, and decrease in progression-free survival in glioma patients. Hence, our study may forecast the potential application of VPA for phase I clinical trial. Nevertheless, there are several limitations to the present study. Firstly, more apoptotic proteins containing p53, p21, cleaved PARP, cleaved caspase9, p-JNK, and p-ERK should be detected in glioma cells exposed to VPA. Secondly, in autophagy assessments, some direct detection methods for autophagosomes, especially transmission electron microscopy, need to be adopted. Thirdly, other autophagic signaling pathways, including Beclin-1, p53, ROS, and AMPK, should be evaluated. Last but not least, the definite mechanism between apoptosis and autophagy promoted by VPA needs further investigation.

In conclusion, our current study verified that VPA promoted cellular apoptosis and autophagy by activating caspase3, Bax/Bcl-2, and Akt/mTOR signaling in glioma. Furthermore, autophagy induction by VPA was revealed as a mechanism in apoptosis initiated by VPA, which may provide a basis for using VPA in the subsequent experiments.

Statement of Human and Animal Rights
This article does not contain any studies with human or animal subjects.

Statement of Informed Consent
There are no human subjects in this article and informed consent is not applicable.

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