

## **Glibenclamide induces apoptosis by activating reactive oxygen species dependent JNK pathway in hepatocellular carcinoma cells**

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**Running title: Glibenclamide induces HCC cell apoptosis through ROS/JNK pathway**

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## **Abstract**

Glibenclamide (Gli) is a widely employed drug in the treatment of type 2 diabetes and many lines of evidence has described its anti-tumoral effects in some neoplasms. The aim of the present study was to investigate the effect of Gli on apoptosis of human hepatocellular carcinoma (HCC) cells and to analyze the underlying pathway involved in this action. Two HCC cell lines, HepG-2 and Huh7 were used as the cell models. We found that Gli treatment significantly inhibited cell viability, induced a significant cell-cycle arrest in G2/M phase and induced apoptosis in both HepG-2 and Huh7 cells. We further verified that apoptosis induction by Gli was accompanied by increase of ROS levels and activation of the JNK pathway. Scavenging of the intracellular ROS with its blocker N-acetyl-cysteine (NAC) could mitigate the Gli-induced apoptosis and JNK activation in the two HCC cell lines. Furthermore, inhibition of JNK pathway by its inhibitor SP100625 effectively reduced Gli-induced apoptosis in HCC cells. In conclusion, Gli treatment significantly induced cell apoptosis by promoting ROS dependent JNK pathway activation in HCC cells. Gli may be a potential clinical anti-tumor drug for HCC.

**Keywords:** Glibenclamide; Hepatocellular carcinoma; Apoptosis; Reactive oxygen species; JNK pathway

## **Introduction**

Hepatocellular carcinoma (HCC) is one of the most prevalent malignancies and the third most common cause of cancer-related deaths in the world, accounting for 80%~90% of all primary liver cancers [1]. The global incidence of HCC is predicted to keep rising over the next few years, and studies for exploring more effective treatment options are quite urgent.

The combination of unrestrained cell proliferation and impaired apoptosis plays a major role in the progression of HCC tumorigenesis. Apoptosis, also known as programmed cell death, maintains the healthy survival/death balance in metazoan cells. It is executed by apoptotic proteins including released cytochrome c (Cyt-c) and the activated caspase-3 (cleaved caspase-3) through either extrinsic or intrinsic pathways [2]. Morphological hallmarks of apoptosis include cell shrinkage, chromatin condensation and DNA fragmentation [3]. Apoptosis is tightly controlled, while defective apoptosis renders cancer cell resistant to treatment and promotes carcinogenesis.

Sulphonylureas, through directly acting on pancreatic  $\beta$  cells to increase insulin secretion and lower blood glucose concentrations, are widely used in treatment of type

2 diabetes. Mechanistically, these drugs bind to the  $\beta$  cell sulphonylurea receptor (SUR), a regulatory subunit of ATP-sensitive potassium channels ( $K_{ATP}$  channels), and close  $K_{ATP}$  channels to reduce cellular potassium efflux thus favor membrane depolarization, the induction of  $Ca^{2+}$  influx, and insulin secretion [4-6]. Glibenclamide (Gli, also known as Glyburide) is a second generation sulphonylurea, and is widely employed in the treatment of diabetic patients [7,8]. As is known, Gli can block mitochondrial  $K_{ATP}$  channels, which play an important role in production of intracellular reactive oxygen species (ROS) [9]. ROS activates both pro- and anti-tumorigenic signaling in cancer cells to control cell growth and apoptosis [10]. This clue raises the possibility that Gli might impact on cancer cell survival through ROS generation. Now increasing evidence reveals that Gli exerts anti-tumoral effects in many neoplastic cell lines [11,12]. Its genotoxicity in cancer cells or normal cells are heated discussed in recent years, both in vitro and in vivo [13-15].

Considering the anti-tumoral effects of Gli on various cancers, we speculated that Gli might also impact on HCC cell survival, which has not been addressed before. As an oral anti-diabetic agent, Gli needs to be metabolized by the liver. Therefore, investigating the effects of Gli on HCC cells is of important guiding significance for the medication of clinical diabetic patients combination with HCC.

## **Materials and methods**

### **Cell culture and reagents**

Human HCC cells HepG-2 and Huh7 cells were from the Cell Resource of Shanghai

Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China).

Cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS, 2 mM L-glutamine and PS (100 U/ml and 100 µg/ml) at 37°C in an atmosphere of 5% CO<sub>2</sub>. Glibenclamide (Gli) and N-acetyl-L-cysteine (NAC) were purchased from Sigma (St. Louis, MO, USA). JNK inhibitor SP600125 was purchased from Selleck (Houston, TX, USA).

### **Cell counting kit (CCK)-8 assay**

Inhibition of cell viability by Gli was measured by using CCK-8 (Dojindo, Kumamoto, Japan). Cells were seeded in a 96-well-plate at a density of 5,000 cells per well containing 100 µL medium. After routinely incubation overnight, cells were treated with various concentrations of Gli for another 24 hrs. Then cells were incubated with CCK-8 working solution for 2 hrs following the manufacturer's instruction. The resulting absorbance at 450 nm was measured on a microplate reader. Three independent experiments were performed.

### **Hoechst 33342 staining**

Hoechst 33342 staining was carried out to observe morphological characteristics of apoptotic cells. Briefly, cells were seeded on coverslips in 24-well plates at a density of  $5 \times 10^4$ /ml per well and incubated overnight. Then cells were treated with 100 µM Gli for another 24 hrs. After removal of the medium, cells were washed twice with PBS, fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.1% Triton X-100, and stained with Hoechst 33342 solution (5 µg/ml) for 10 min at room temperature in

the dark. Subsequently the slides were rinsed twice with PBS and photographed with a fluorescence microscopy (Olympus Optical Co., Ltd., Tokyo, Japan) for morphological changes of the nucleus.

### **Cell cycle analysis**

To investigate the cell cycle attribution influenced by Gli, cells pretreated with Gli (100  $\mu$ M) for 24 hrs were trypsinized, washed with PBS, fixed with ice cold 70% ethanol and incubated overnight at -20°C. Then cells were washed with PBS and stained in 0.5 ml of propidium iodide (PI) staining solution (50  $\mu$ g/ml PI in PBS containing 0.2 mg/ml of DNase-free RNase A) for 1 hr in darkness at 37 °C. Finally the cells were analyzed by a flow cytometer (Becton Dickinson, USA).

### **Measurement of intracellular ROS generation**

Intracellular ROS level was determined by using the Reactive Oxygen Species Assay Kit (Beyotime, Shanghai, China) in which the positive signals of 2',7'-dichlorfluorescein-diacetate (DCFH-DA) probes reflected ROS generation. Briefly, cells were seeded in a 6-well plate at a density of  $5 \times 10^5$ /ml and exposed to Gli (100  $\mu$ M) for 12 h. According to the protocol provided by the manufactory, cells were incubated with 10  $\mu$ M DCFH-DA solution at 37°C for 30 min in darkness, followed by three washes with PBS and flow cytometry analysis of 2',7'-dichlorfluorescein (DCF) fluorescence.

### **Western blotting**

Cells were lysed with RIPA buffer (Beyotime, Shanghai, China) containing protease

inhibitor cocktail (Sigma) for 30 minutes on ice, followed by centrifugation at 13,000 rpm for 15 minutes at 4°C. The supernatant was collected and protein concentration was determined by BCA Protein Assay Kit (Beyotime). Equal amounts of whole cell extracts were heated with SDS sample buffer (Beyotime), loaded onto the 10% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membranes were then blocked and incubated overnight with primary antibodies at 4°C individually. Immunoreactivity was detected by using horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG, and visualized by chemiluminescence. The antibodies were purchased from the following companies:  $\beta$ -actin and the secondary antibodies (goat anti-rabbit and goat anti-mouse; Santa Cruz, Heidelberg, Germany); JNK, p-JNK, uncleaved and cleaved caspase-3/PARP (Cell Signaling, Frankfurt, Germany).

### **Annexin V/PI staining**

Apoptosis induced by Gli was examined by using the Annexin V-FITC Apoptosis Detection Kit according to the manufacturer's protocol (KeyGENE, Nanjing, China). Briefly, cells were trypsinized, washed with cold PBS, pelleted and stained using the Annexin V-FITC reaction reagent at room temperature for 20 min in darkness. Then, samples were analyzed by a flow cytometer (Becton Dickinson, USA).

### **Statistics**

Data are presented as mean  $\pm$  S.D. of at least three independent experiments and analyzed using Graphpad Prism 5.0 (Graphpad Software Inc., La Jolla, CA). Student's

*t*-test was used for comparison between two groups.  $P < 0.05$  was considered to be statistically significant.

## **Results**

### **Gli inhibits HCC cell viability and induces G2/M arrest in HCC cells**

In order to verify the effects of Gli on HCC cell survival, two HCC cell line, HepG-2 and Huh7 were treated with different concentrations of Gli for 24 hrs. Cell viability was assessed by CCK-8 assay. Figure 1A indicates that Gli significantly reduced cell viability in a dose-dependent manner as compared to that noted in the control cells (vehicle treatment with 0.1% DMSO). We observed a significant inhibition on cell viability when Gli was higher than 50  $\mu\text{M}$  for Huh7 cells, and over 100  $\mu\text{M}$  for HepG-2 cells ( $P < 0.01 \sim 0.05$ ). The analysis of cell cycle distribution demonstrated that Gli produced a significant arrest in the G2/M phase at 24 hrs post treatment ( $P < 0.05$ ; Figure 1B and 1C).

### **Gli induces cell apoptosis in HCC cells**

To determine if the decrease in cell viability exerted by Gli could be due to apoptosis, we detected cell apoptosis by employing Hoechst 33342 staining. As shown in Figure 2A, Gli-treated cells exhibited different degrees of cell shrinkage, chromatin condensation and nuclei fragmentation in HepG-2 and Huh7 cells. The quantification result indicated that Gli induced a significant cell apoptosis ( $P < 0.01$ ; Figure 2B).

### **Gli increases intracellular ROS level and activates JNK pathway**

As mentioned above, through blocking mitochondrial  $K_{ATP}$  channels, Gli plays an important role in production of intracellular ROS, which is an important regulator in various pathways, including apoptosis, and also promotes the sustained JNK activation [16,17]. To investigate whether ROS level was elevated by Gli in HCC cells, DCFH-DA, a fluorescence probe was used to monitor intracellular ROS generation. As shown in Figure 3A and 3B, cells treated with Gli exhibited a dramatic increase of ROS level compared with control, which was reversed by the ROS scavenger, NAC ( $P<0.05$ ). To further investigating the mechanism of the pro-apoptosis activity of Gli, we analyzed the effects of Gli on JNK pathway. Figure 4A showed that Gli increased the phosphorylation of JNK in a time-dependent manner. The expression of cleaved caspase-3 or PARP was also upregulated after 4 or 8 hrs of Gli exposure, respectively, leaving the decreased amount of uncleaved caspase-3 or PARP, which indicated the occurrence of mitochondrial apoptosis (Figure 4A). We further found that pretreatment with ROS scavenger, NAC, significantly reversed the phosphorylation of JNK, cleaved caspase-3 and PARP in HepG-2 cells (Figure 4B).

### **ROS/JNK pathway mediates cell apoptosis induced by Gli**

To determine whether Gli-induced apoptosis has a connection with ROS generation, we used NAC, the ROS blocker, for apoptosis investigation. Strikingly, Annexin V/PI staining coupled with flow cytometry analysis revealed that Gli significantly induced HCC cell apoptosis, however, pretreatment of NAC could reverse the apoptosis induced by Gli ( $P<0.05$ ; Figure 5A and 5B). On the other hand, treatment of JNK inhibitor SP600125 obviously reduced the levels of phosphorylation of JNK, cleaved caspase-3

and PARP (Figure 6A). Consistent with this, Gli combined with SP600125 treatment lowered the apoptosis rate than Gli alone ( $P<0.01$ ; Figure 6B and 6C). All the above results suggest that HCC cell apoptosis induced by Gli could be mediated by the ROS/JNK pathway.

## **Discussion**

Gli is a frequently used medicine treating type 2 diabetes and well recognized for its anti-proliferation effects in multiple cancers. For instance, it has been previously demonstrated that Gli inhibits cell growth and by inducing G1 arrest in human breast cancer cells [12]. In gastric cancer, Gli induces decline of cell viability, accompanied with cell apoptosis, ROS generation and JNK activation [11]. There's still none report expounding the effects of Gli on HCC cell survival except that, Gli induces apoptosis through inhibition of cystic fibrosis transmembrane conductance regulator (CFTR) Cl(-) channels and intracellular Ca(2+) release in HepG-2 cells [18]. In the present study, we strengthened that Gli could inhibit cell viability, cause G2/M arrest, and induce apoptosis in HCC cells. More importantly, we verified that Gli induced apoptosis by activating ROS/JNK pathway.

One of the key findings of the Gli-induced apoptosis in HCC cells is the generation of ROS. ROS forms as natural byproduct of the metabolism of oxygen and plays critical roles in inducing cell apoptosis [19]. Our results indicated that Gli caused a significant elevation of intracellular ROS level. However, the ROS scavenger, NAC, obviously repressed the ROS, JNK pathway and apoptosis induced by Gli. This finding illustrated

that Gli triggered apoptosis by induction of ROS/JNK pathway.

In HCC cells, we found that Gli treatment increased JNK phosphorylation in a time-dependent manner. Since JNK is generally responsible for the apoptotic response induced by extracellular DNA-damaging agents [20], we employed JNK inhibitor SP600125 to observe the Gli's cytotoxicity. The results showed that SP600125 markedly inhibited Gli-induced apoptosis, accompanied by the reversion of cleaved caspase-3 and PARP. These findings demonstrated that ROS-dependent JNK activation mediated the Gli-induced apoptosis in HCC cells. Thus, the increase of ROS level and JNK activation may naturally sensitize HCC cells to Gli. Application of NAC, the ROS scavenger, could effectively eliminated the ROS/JNK activation and protect HCC cells from Gli-induced cell apoptosis. This is the first work to show the activation of ROS/JNK pathway by Gli treatment in HCC cells, and identifying candidate agents activating ROS/JNK pathway may be a new strategy for HCC chemotherapy.

In conclusion, our study showed that Gli inhibited cell viability by causing G2/M arrest and inducing apoptosis in human HCC cells. Additionally, the pro-apoptosis effect of Gli is mediated by the activation of ROS/JNK pathway. Although in vivo data and more detailed mechanisms are still absent from the present study, our data provides further insights into the anti-tumoral effects of Gli in HCC cells, and proposes that Gli may be used as a new anti-cancer drug for HCC.

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## **Disclosure**

Competing Interests: The authors declare no conflict of interest.

## **Author contribution**

Bin Yan, Zhiyong Peng, Xiao Xing, Chunling Du designed the research; Bin Yan and Zhiyong Peng performed the experiments; Xiao Xing and Chunling Du analyzed the data; Bin Yan and Chunling Du wrote the paper.

## **References**

- 1 Jemal, A., Bray, F., Center, M.M., Ferlay, J., Ward, E., Forman, D. (2011) Global cancer statistics. *CA Cancer J Clin.* **61**, 69-90.
- 2 Hassan, M., Watari, H., AbuAlmaaty, A., Ohba, Y., Sakuragi, N. (2014) Apoptosis and molecular targeting therapy in cancer. *Biomed Res Int.* **2014**, 150845.
- 3 Fulda, S., Debatin, K.M. (2004) Apoptosis signaling in tumor therapy. *Ann N Y Acad Sci.* **1028**, 150-156.
- 4 Krentz, A.J., Bailey, C.J. (2005) Oral antidiabetic agents: current role in type 2 diabetes mellitus. *Drugs.* **65**, 385-411.
- 5 Lebovitz, H.E. (2002) Treating hyperglycemia in type 2 diabetes: new goals and strategies. *Cleve Clin J Med.* **69**, 809-820.
- 6 Lebovitz, H.E. (2004) Oral antidiabetic agents: 2004. *Med Clin North Am.* **88**, 847-863.
- 7 Groop, L.C. (1992) Sulfonylureas in NIDDM. *Diabetes Care.* **15**, 737-754.

- 8 Riddle, M.C. (2003) Editorial: sulfonylureas differ in effects on ischemic preconditioning--is it time to retire glyburide? *J Clin Endocrinol Metab.* **88**, 528-530.
- 9 Pain, T., Yang, X.M., Critz, S.D., Yue, Y., Nakano, A., Liu, G.S., Heusch, G., Cohen, M.V., Downey, J.M. (2000) Opening of mitochondrial K(ATP) channels triggers the preconditioned state by generating free radicals. *Circ Res.* **87**, 460-466.
- 10 Moloney, J.N., Cotter, T.G. (2017) ROS signalling in the biology of cancer. *Semin Cell Dev Biol.* **S1084-9521**, 30383-30384.
- 11 Qian, X., Li, J., Ding, J., Wang, Z., Duan, L., Hu, G. (2008) Glibenclamide exerts an antitumor activity through reactive oxygen species-c-jun NH2-terminal kinase pathway in human gastric cancer cell line MGC-803. *Biochem Pharmacol.* **76**, 1705-1715.
- 12 Núñez, M., Medina, V., Cricco, G., Croci, M., Cocca, C., Rivera, E., Bergoc, R., Martín, G. (2013) Glibenclamide inhibits cell growth by inducing G0/G1 arrest in the human breast cancer cell line MDA-MB-231. *BMC Pharmacol Toxicol.* **14**, 6.
- 13 Wonderlin, W.F., Strobl, J.S. (1996) Potassium channels, proliferation and G1 progression. *J Membr Biol.* **154**, 91-107.
- 14 Pardo, L.A. (2004) Voltage-gated potassium channels in cell proliferation. *Physiology (Bethesda).* **19**, 285-292.
- 15 de Sant'Anna, J.R., Franco, C.C., Mathias, P.C., de Castro-Prado, M.A. (2015) Assessment of in vivo and in vitro genotoxicity of glibenclamide in eukaryotic cells. *PLoS One.* **10**, e0120675.
- 16 Simon, H.U., Haj-Yehia, A., Levi-Schaffer, F. (2000) Role of reactive oxygen

species (ROS) in apoptosis induction. *Apoptosis*. **5**, 415-418.

17 Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., Karin, M. (2005) Reactive oxygen species promote TNF $\alpha$ -induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell*. **120**, 649-661.

18 Kim, J.A., Kang, Y.S., Lee, S.H., Lee, E.H., Yoo, B.H., Lee, Y.S. (1999) Glibenclamide induces apoptosis through inhibition of cystic fibrosis transmembrane conductance regulator (CFTR) Cl<sup>-</sup> channels and intracellular Ca<sup>2+</sup> release in HepG-2 human hepatoblastoma cells. *Biochem Biophys Res Commun*. **261**, 682-688.

19 Trachootham, D., Alexandre, J., Huang, P. (2009) Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? *Nat Rev Drug Discov*. **8**, 579-591.

20 Singh, A.V., Bandi, M., Raje, N., Richardson, P., Palladino, M.A., Chauhan, D., Anderson, K.C. (2011) A novel vascular disrupting agent plinabulin triggers JNK-mediated apoptosis and inhibits angiogenesis in multiple myeloma cells. *Blood*. **117**, 5692-5700.

## Figure legends

**Figure 1. Effects of Gli on HCC cell viability and cell cycle progression. A:** HepG-2 and Huh7 cells were treated with different concentrations of Gli (0, 10, 50, 100, 500, 1000  $\mu$ M dissolved in DMSO) for 24hrs. Cell viability was determined by the CCK-8 assay. \*P<0.05, \*\*P<0.01. **B:** HepG-2 and Huh7 cells were treated with Gli (100  $\mu$ M) for 24 hrs and cell cycle was analyzed by flow cytometry. Gli caused a G2/M arrest. **C:**

Quantification for cell cycle analysis. The percentage of cell population at G0/G1, S, and G2/M phases are represented as mean  $\pm$  S.D. of three independent experiments. \* $P$ <0.05.

**Figure 2. Gli induced cell apoptosis in HCC cells.** **A:** Apoptotic nuclear morphological changes were evaluated by Hoechst 33342 staining and observed under a fluorescent microscopy. **B:** The quantification result of apoptotic cells in **A**. The percentage of apoptotic cells was calculated as (highlighted blue staining cell counts) / (total cell counts)  $\times$  100. \*\* $P$ <0.01.

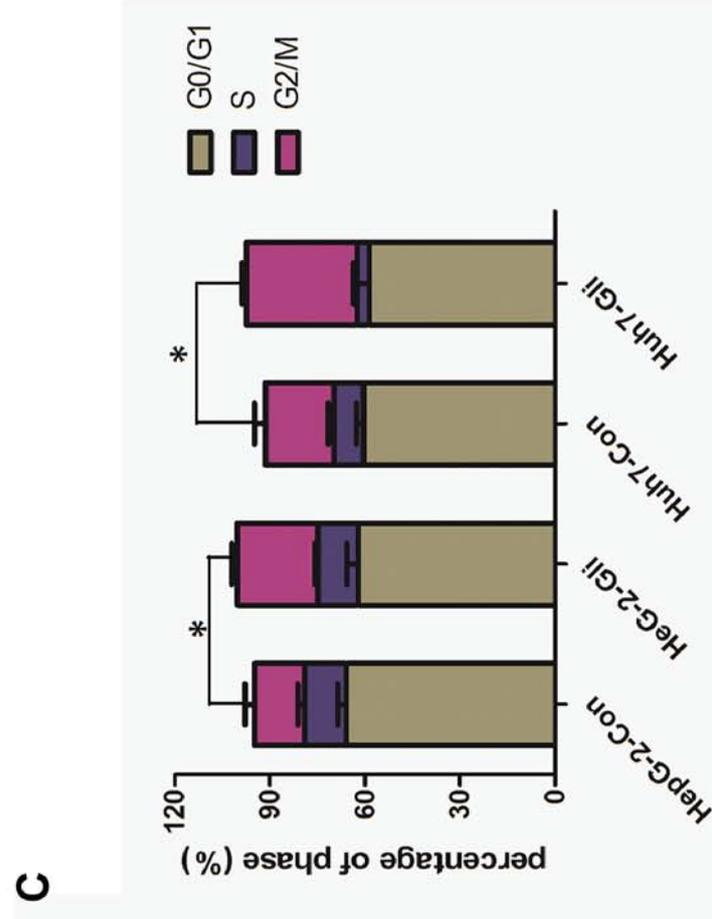
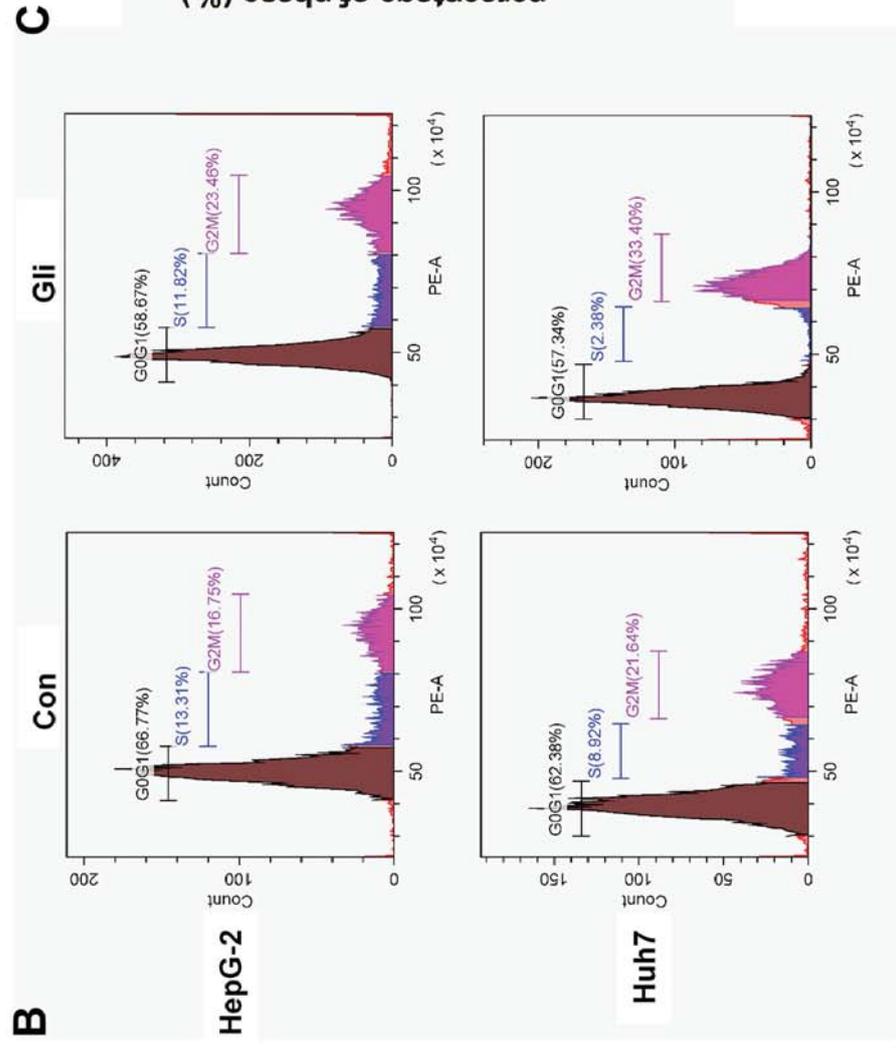
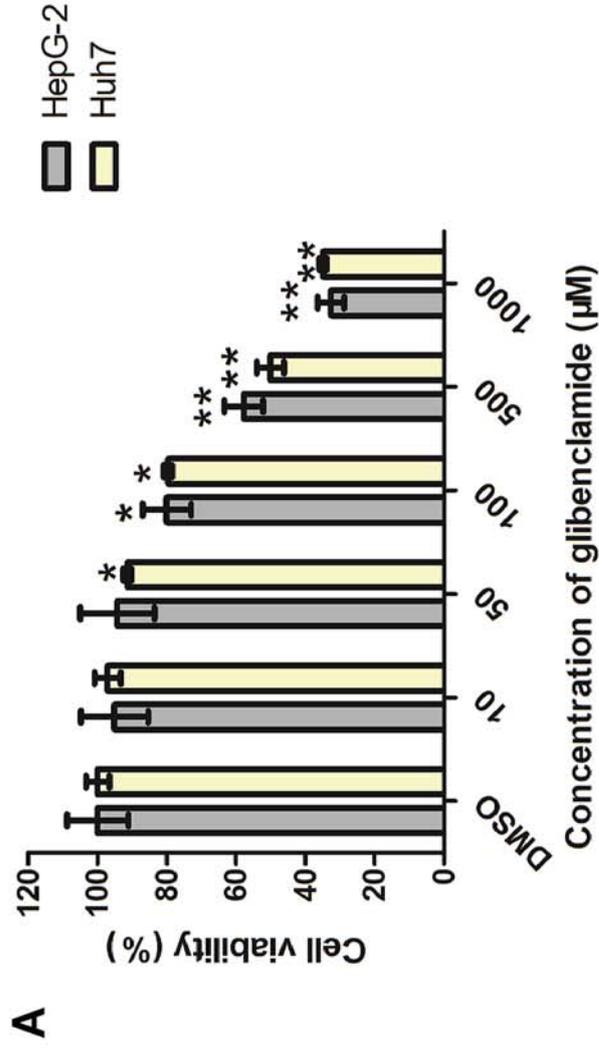
**Figure 3. Gli treatment led to intracellular ROS generation.** **A:** HepG-2 and Huh7 cells were treated with Gli (100  $\mu$ M) in presence or absence of NAC (5 mM) for 24 hrs and intracellular ROS levels were analyzed by DCFH-DA coupled with flow cytometry. Gli caused elevation of ROS levels and NAC pretreatment blocked this action. **B:** The mean fluorescent intensity of ROS was shown in histograms. Data are presented as means  $\pm$  S.D. (n=3). \* $P$ <0.05.

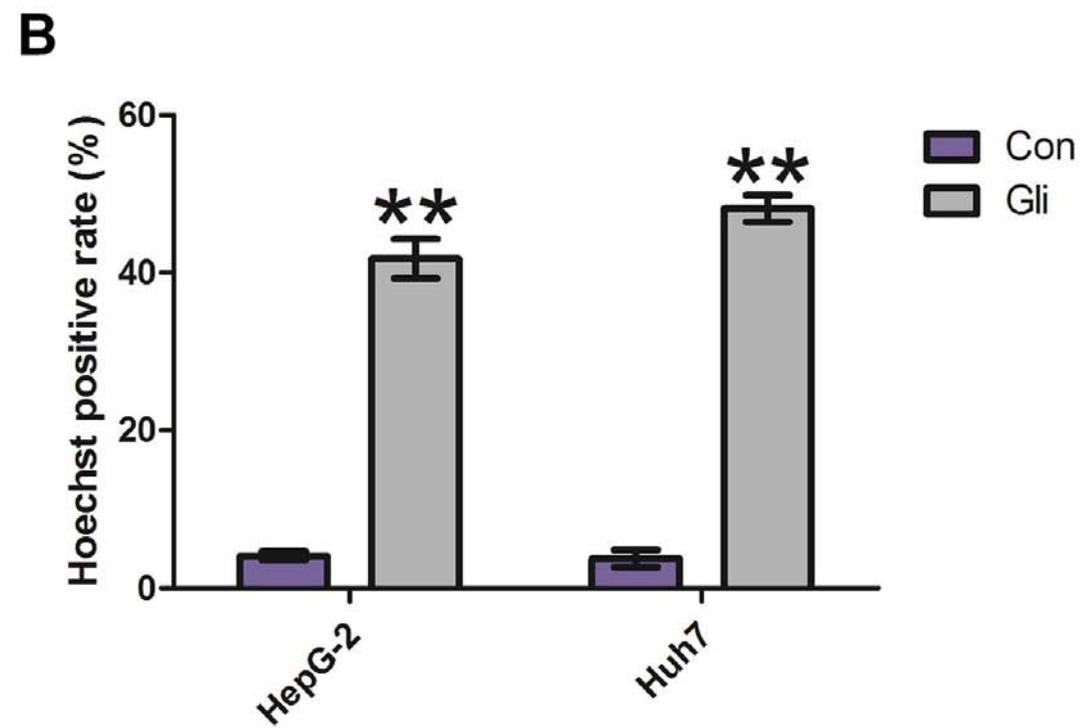
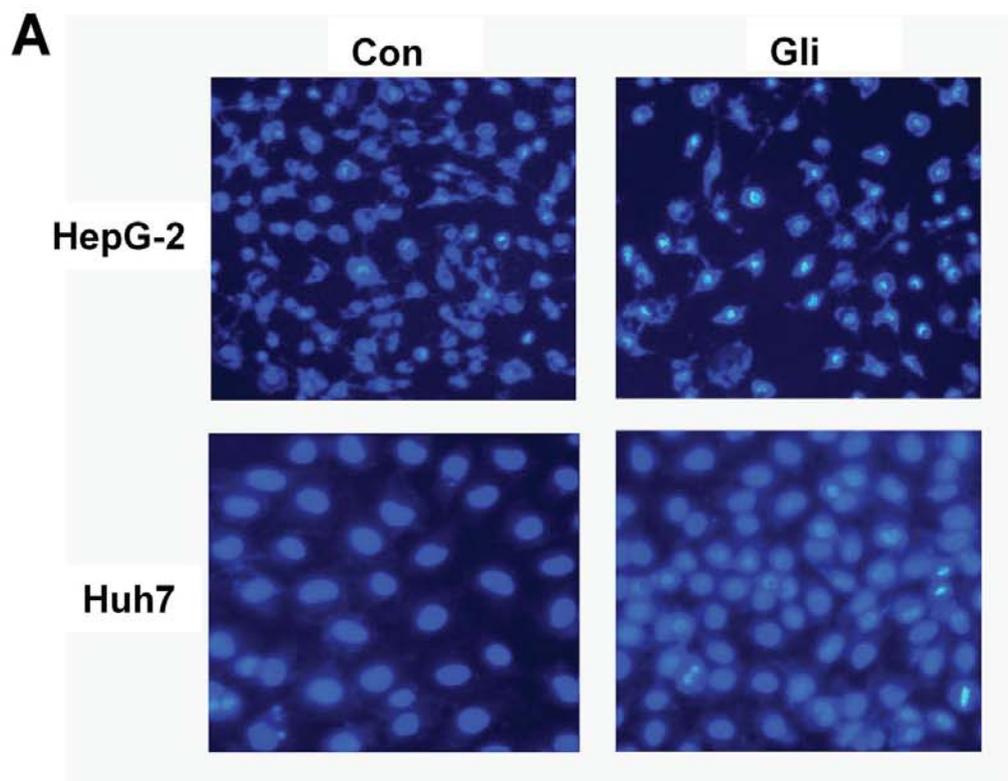
**Figure 4. Gli activated JNK pathway in HCC cells.** **A:** HepG-2 cells were treated with Gli (100  $\mu$ M) for indicated hours. Levels of p-JNK, JNK, uncleaved caspase-3, cleaved caspase-3, uncleaved PARP, cleaved PARP and  $\beta$ -actin were analyzed by western blotting. **B:** HepG-2 cells were treated with Gli (100  $\mu$ M) in presence or absence of NAC (5 mM) for 24 hrs. Levels of p-JNK, JNK, cleaved caspase-3, cleaved PARP and  $\beta$ -actin were analyzed by western blotting.

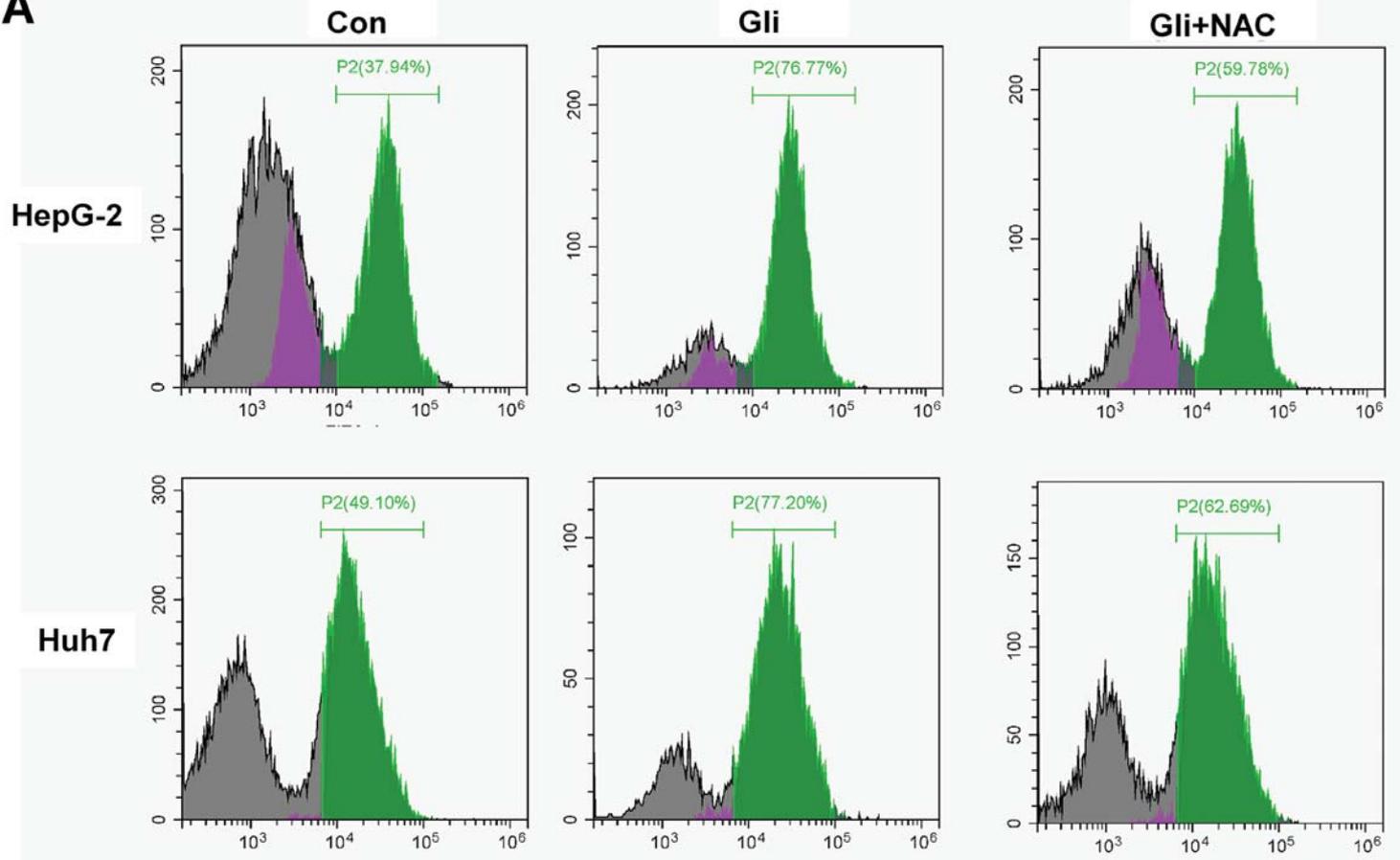
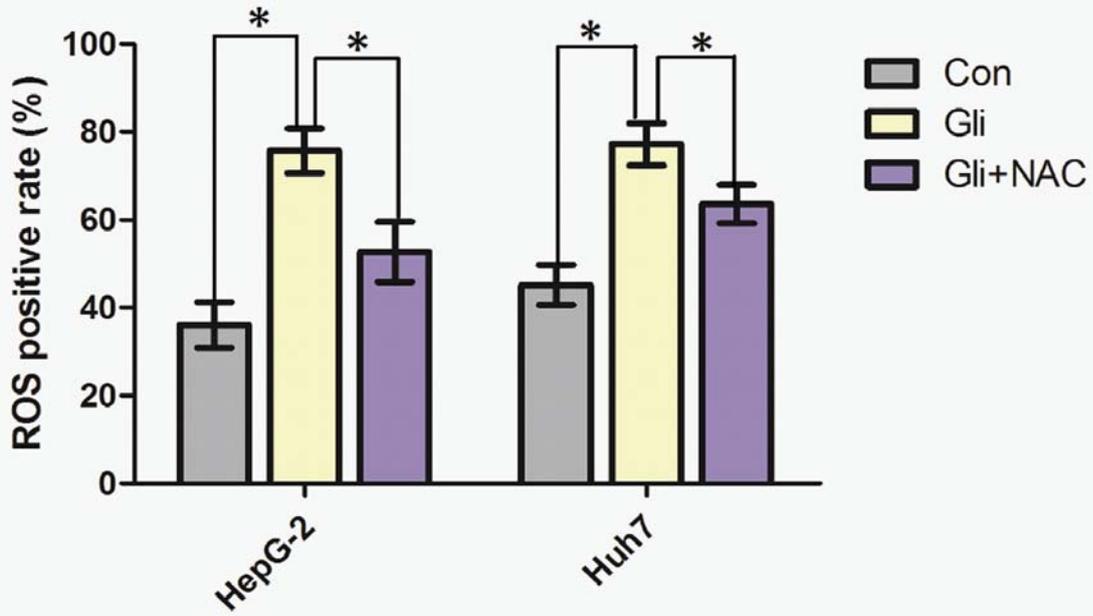
**Figure 5. Promotion of cell apoptosis by Gli was ROS dependent.** **A:** HepG-2 and Huh7 cells were pretreated with NAC (5 mM) for 2 hrs and then treated with Gli (100

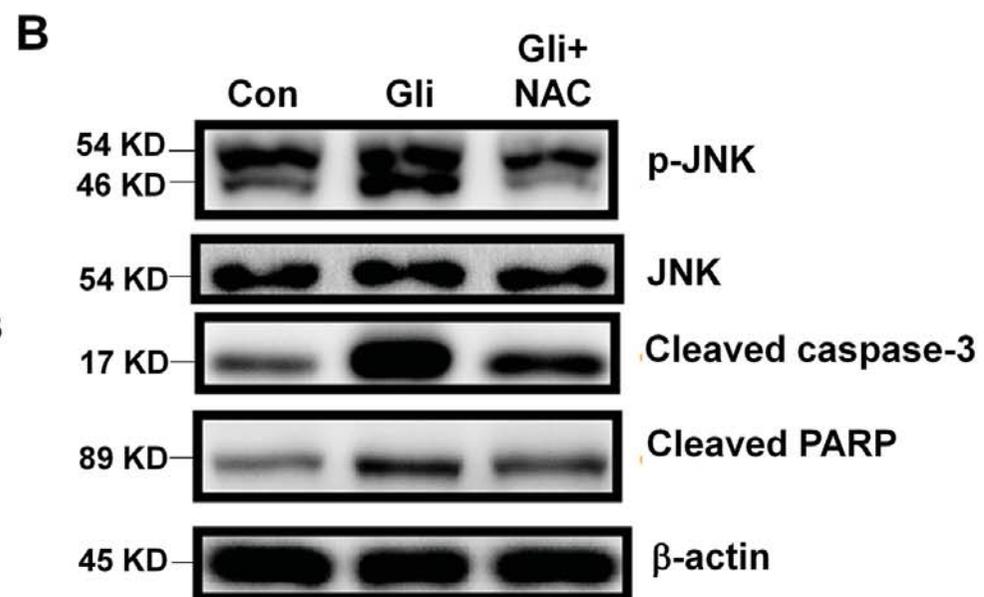
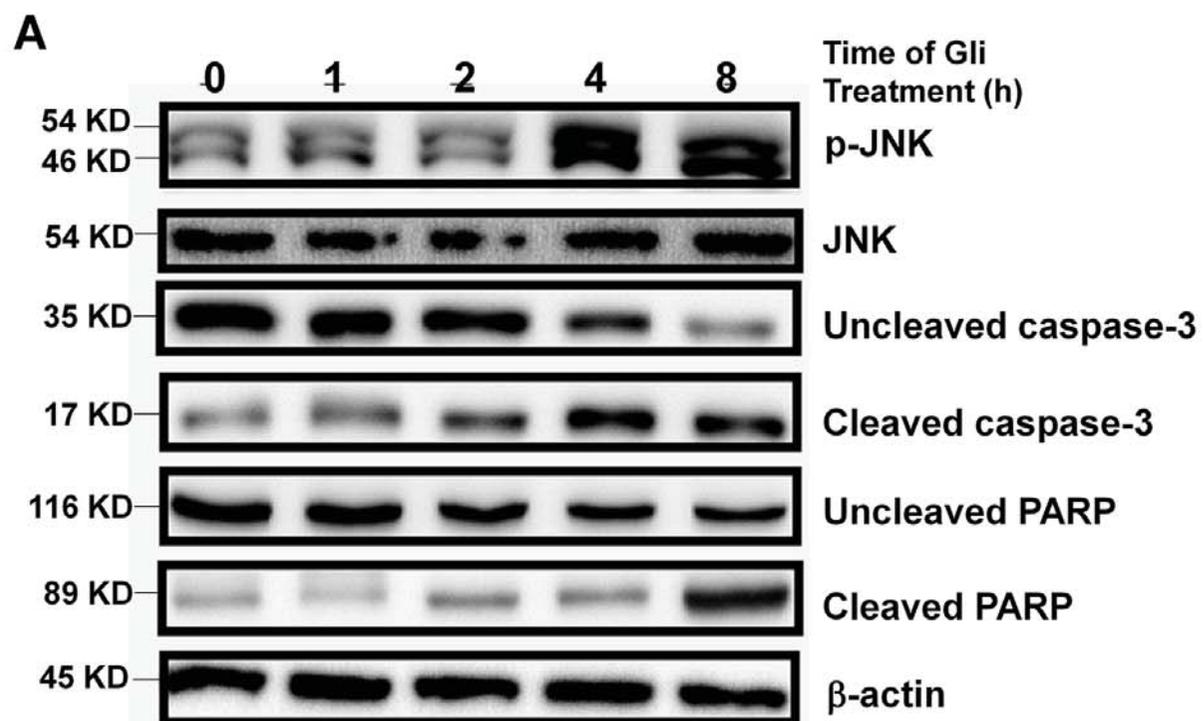
$\mu\text{M}$ ) for 24 hrs. Cell apoptosis was analyzed by Annexin V/PI staining and flow cytometry. **B:** Quantitative analysis of apoptosis rate was shown in histograms. Data are presented as means  $\pm$  S.D. (n=3). \* $P$ <0.05.

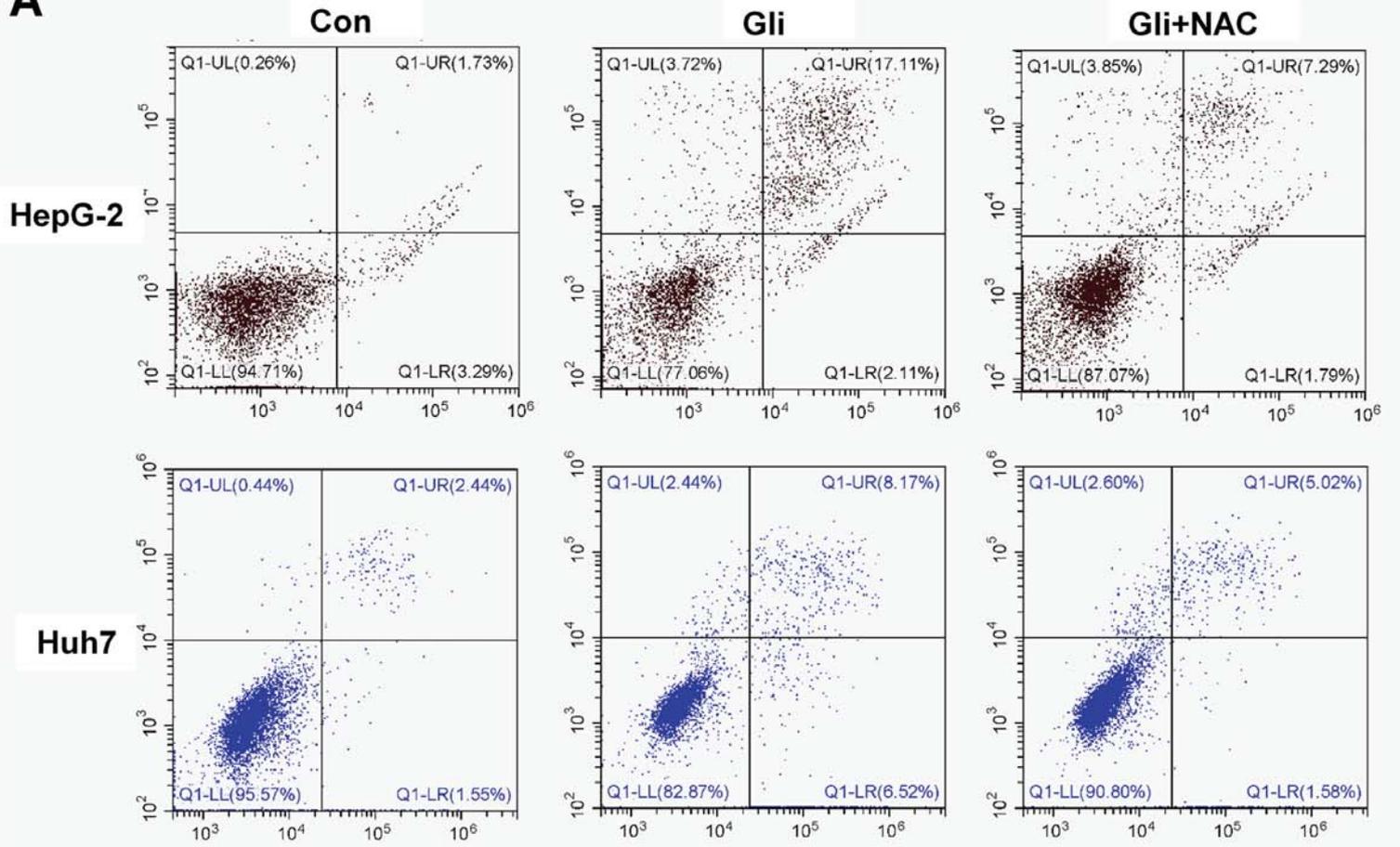
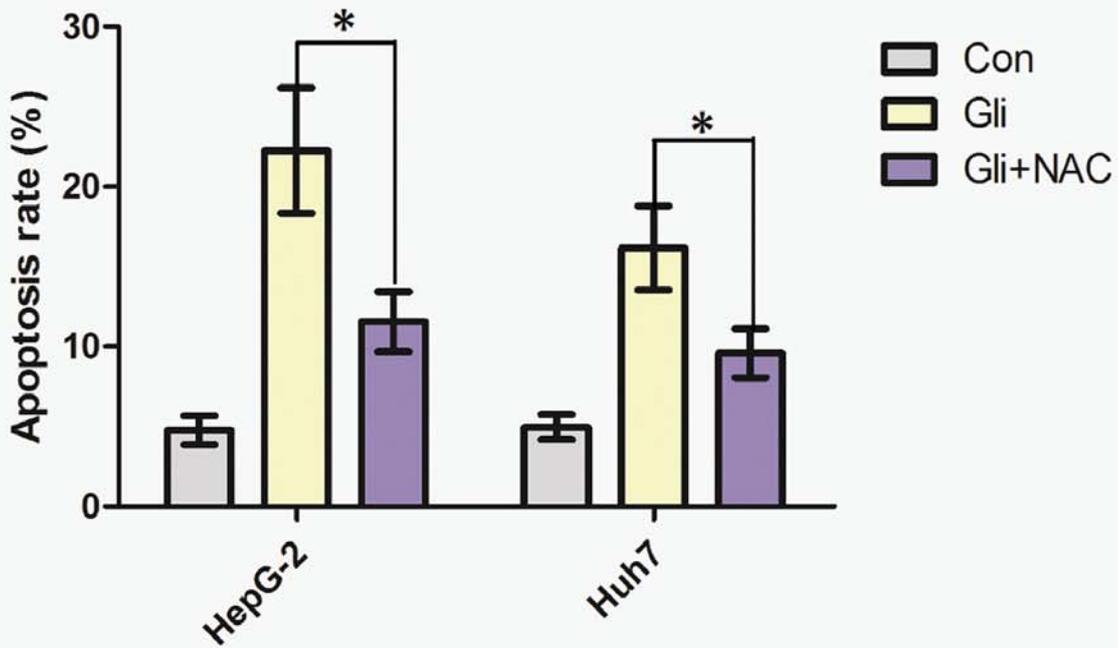
**Figure 6. Induction of cell apoptosis by Gli was mediated by JNK activation. A:** HepG-2 and Huh7 cells were treated with Gli alone or combined with SP100625 (50 nM) for 8 hrs. Levels of p-JNK, JNK, cleaved caspase-3, cleaved PARP and  $\beta$ -actin were analyzed by western blotting. **B:** Cell apoptosis was analyzed by Annexin V/PI staining and flow cytometry. **C:** Quantitative analysis of apoptosis in **B** was shown in histograms. Data are presented as means  $\pm$  S.D. (n=3). \* $P$ <0.05.



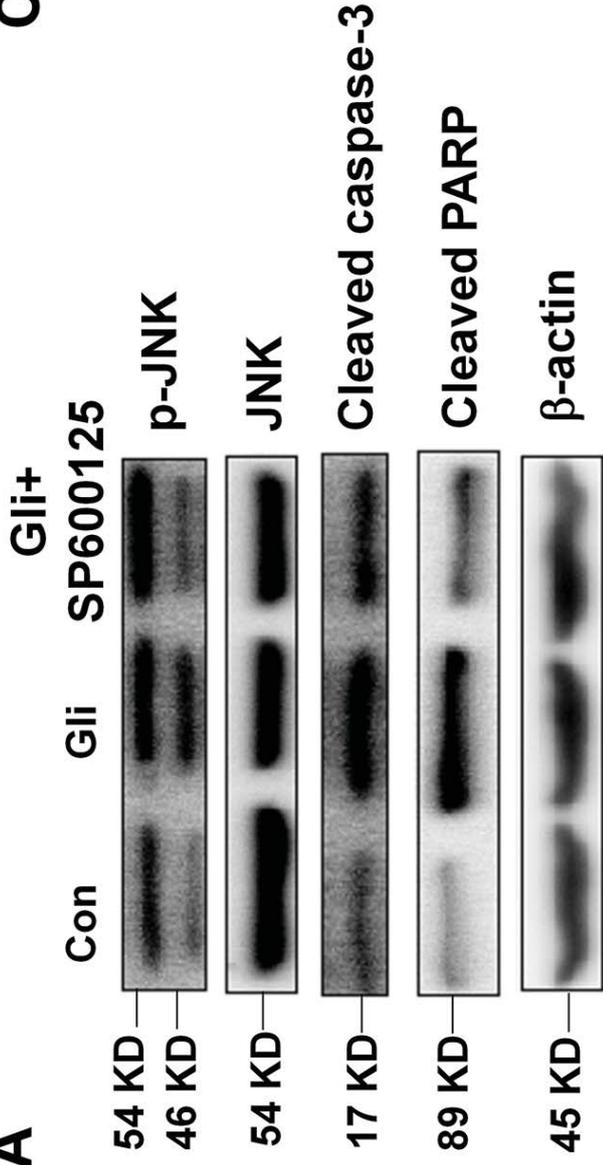


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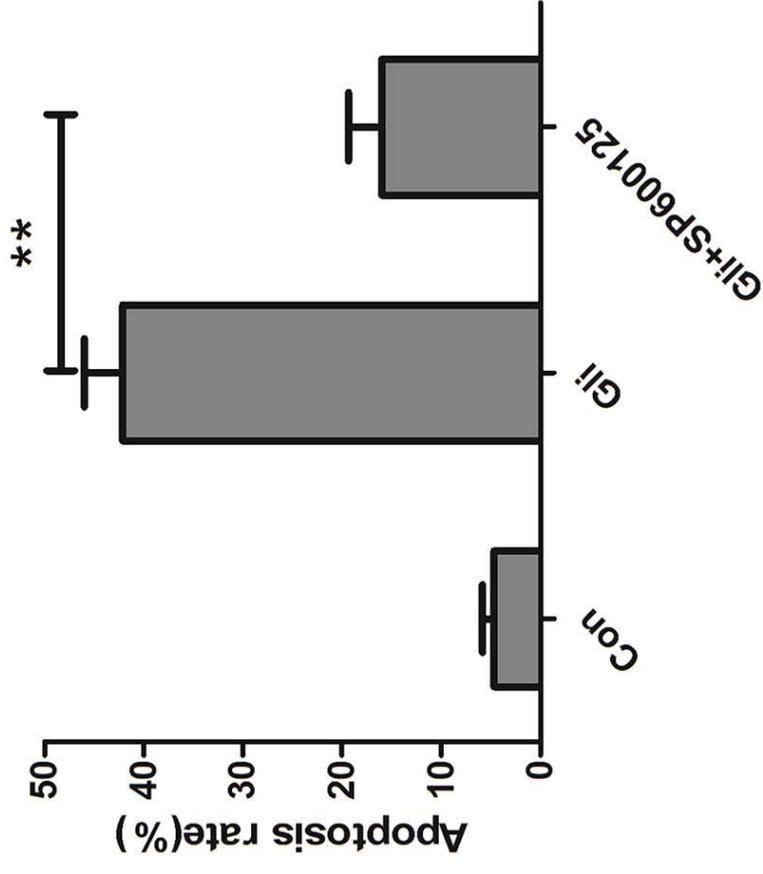


**A****B**

**A**



**C**



**B**

