GL-V9 reverses adriamycin resistance in hepatocellular carcinoma cells by affecting JNK2-related autophagy

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[ABSTRACT] Adriamycin resistance in HCC seriously hinders the treatment of patients, it is necessary to investigate the mechanisms. Autophagy is involved in adriamycin resistance and JNK2 is related to autophagy. However, whether JNK2 inducing drug resistance though autophagy is unknown. GL-V9, a new synthesized flavonoid derivative, has been proved of its anti-tumor effects. The aim of the study is to explore the role of JNK2-related autophagy on adriamycin-induced drug resistance and the effects of GL-V9 on reversing adriamycin resistance. We concluded that JNK2 played an important role in drug resistance induced by adriamycin. The high expression of JNK2 activated protective autophagy in Hep G2-DOXR cells under non-stress condition, which protected cells from drug attacking. Furthermore, we found that GL-V9 reversed adriamycin resistance by blocking the JNK2-related protective autophagy in HCC.

[KEY WORDS] GL-V9; Drug resistance; JNK2; Autophagy

[Introduction] Hepatocellular carcinoma (HCC) is the fifth most common tumor all over the world, and it is the third most common cause of cancer-related mortality [1-3]. With insidious onset and rapid invasive growth, the treatment of liver cancer is difficult. Surgery, combined with radiotherapy and chemotherapy, is the first choice and the most effective method for the treatment of liver cancer. However, persistent and effective chemotherapy drugs are rare.

Adriamycin is a representative broad spectrum anticancer drug, which is widely used in various types of cancer, such as acute leukemia, malignant lymphoma, breast cancer, ovarian cancer, gastric cancer, liver cancer and so on. The anthracycline adriamycin is one of the most commonly used drugs in clinical treatment [4]. It is one of the most widely used anti-HCC drugs systemically or locally [4]. However, HCC can eventually develop chemoresistance during long-term adriamycin chemotherapy, resulting in recurrence and poor prognosis [5]. Adriamycin resistance in HCC seriously hinders the treatment of patients. Therefore, it is necessary to investigate the mechanism of adriamycin resistance in HCC.

Autophagy is essential for maintaining cellular energy homeostasis and functions, it is a regulatory mechanism to defend against environmental changes and metabolic responses to stress. Researchers have spent a lot of time exploring the role of autophagy in drug resistance [7-10]. Autophagy plays a dual role in tumor formation and therapy. It is of great significance and clinical value to study the effect of autophagy on adriamycin resistance.

The JNK (‘c-Jun amino-terminal kinase’; stress-activated protein kinase (SAPK)) kinases are a subfamily of the MAPK (mitogen-activated protein kinase) superfamily. JNK is involved in the regulation of many cellular activities [11-13]. JNK has two main isoforms, JNK1 and JNK2. The role of JNK1 and JNK2 in cancer is complicated and controversial. JNK1 represses Lkb-deficiency-induced lung squamous cell carcinoma progression while promotes hepatocellular carcinoma proliferation by autophagy activation [14, 15]. JNK2 promotes carcinogenesis in skin cancer and myeloma while inhibits breast cancer progression via regulation of cell cycle and DNA repair [16-18]. The relationship between JNK2 and drug resistance has not been reported. In addition, studies proved that JNK2 promoted stress-induced mitophagy by targeting the small mitochondrial form of the tumor suppressor...
ARF for degradation and induced autophagy in hepatocytes [19,20].

GL-V9 (5-hydroxy-8-methoxy-2-phenyl-7-(4-pyrrolidin-1-yl) butoxy)-4H-chromen-4-one), a new synthesized flavonoid derivative, has been proven of its anti-tumor effects. Previous reports showed that it induced apoptosis and G2/M cell cycle arrest in human hepatocellular carcinoma Hep G2 cells [21]. It can also inhibit invasion in human breast carcinoma MDA-MB-231 cells and induce apoptosis of breast cancer via disrupting GSK-3β-modulated mitochondrial binding of HKII [22,23]. The latest research proved that non-lethal dose GL-V9 induced mitotic mutation and p53-dependent senescence in T cell malignant tumors [24].

In this study, we explored the effects of JNK2-related autophagy on the adriamycin resistance and studied the effects of the new synthesized flavonoid GL-V9 on reversing adriamycin resistance.

Materials and Methods

Reagents

GL-V9 (5-hydroxy-8-methoxy-7-(4-pyrrolidin-1-yl) butoxy)-4H-chromen-4-one), C_21H_27NO_5, MW 409.47, purity > 99% is a new flavonoid synthesized by Prof. LI Zhi-Yu (China Pharmaceutical University, China). GL-V9 was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) to 100 mmol·L\(^{-1}\) as a stock solution, stored at –80 °C, and freshly diluted with medium before each experiment. The final DMSO concentration did not exceed 0.1% throughout the study (without influences in cell growth).

Adriamycin (MW 543.52, purity > 97%), purchased from Aladdin (Aladdin Biochemical Technology Co., Ltd, Shanghai), was dissolved in distilled water to make a 10 mmol·L\(^{-1}\) stock solution and freshly diluted with medium before experiments.

Dye DAPI was purchased from Invitrogen (Carlsbad, CA, USA). Antibodies against GAPDH, PARP1, LC3 and Beclin1 were purchased from Proteintech (Wuhan Sanying Biotechnology Co., Ltd., Wuhan). The antibody against p62 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody against JNK2 was purchased from Bioworld Technology (Bioworld Technology Inc, MN, USA). Antibodies against Atg5, Atg7, Active Caspase-3, Bel-2 and Bax were purchased from Abclonal Technology (Wuhan, China).

Cell culture

Human hepatoma cell line Hep G2 and human breast cancer cell line MCF-7 (Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco’s Modified Eagle Medium (DMEM, GibCO, Carlsbad, CA). Their drug-resistant counterparts (Hep G2-DOXR and MCF-7-DOXR) were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA). All medium was supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA), 100 U·mL\(^{-1}\) of penicillin, and 100 μg·mL\(^{-1}\) of streptomycin. The medium of drug-resistant cells was supplemented with 1 μg·mL\(^{-1}\) adriamycin. All cell lines were incubated at 37 °C in a humidified atmosphere containing 5% CO\(_2\) and routinely tested for mycoplasma infection. The adriamycin-resistant cell sublines were established by culturing parental cells (Hep G2 and MCF-7) with increasing concentrations of adriamycin (from 0.01 to 10 μmol·L\(^{-1}\)). When cells could survive at any given concentration of adriamycin, they were passaged in concentrations that were 1.5 to 2-fold higher. Cells were obtained after 11 months, which were able to survive in 10 μmol·L\(^{-1}\) adriamycin. They were named as Hep G2-DOXR and MCF-7-DOXR.

Western blot analysis

Cells were washed with cold PBS and lysed in RIPA Lysis buffer (ThermoFisher, USA) containing protease/phosphatase inhibitors. After lysates concentration was determined by BCA protein assay (Pierce, Rockford, IL, USA), equal amount of denatured proteins were subjected to SDS-PAGE gel electrophoresis and then transferred onto a nitrocellulose membrane (BioTrace NT, PallCor, USA), which was blocked by 5% non-fat milk in PBS, following by incubation at 4 °C with specific primary antibodies overnight. Then, membranes were incubated with HRP goat anti-rabbit immunoglobulin G (IgG; H + L) or anti-mouse IgG (H + L) secondary antibody (Biosharp) for 1 h and finally visualized with chemiluminescence (ThermoFisher).

Annexin-V/DAPI double-staining assay

Cells were harvested and stained with the Annexin V from Annexin V/PI Cell Apoptosis Detection Kit (KeyGen Biotechnology) and dye DAPI according to the manufacturer’s instructions. Data were acquired from flow cytometry (Becton Dickinson, CA, USA).

CCK-8 assay

Cells were seeded into 96 - well plates (Boyang, Shanghai, China) 7000 cells/well. The cells were left overnight and were treated with adriamycin, GL-V9 or adriamycin combined with GL-V9. After 24 h, 20 μL of CCK-8 (Nanjing Vazyme Biotechnology Co., Ltd.) was added to each well and incubated for 4 h at 37 °C and 5% CO\(_2\). Plates were shaking for 2 min, and the optical absorbance was recorded at 450 nm. The inhibitory effect of adriamycin on cells was calculated according to the following equation:

\[
\text{Inhibition ratio (\%) = } \frac{(A_{\text{control}} - A_{\text{treated}})(A_{\text{control}} - A_{\text{blank}})}{100}
\]

\(A_{\text{treated}}\): The absorbance value of the treated group containing medium, cells, drugs to be tested and CCK-8.

\(A_{\text{control}}\): The absorbance value of the control group containing medium, cells and CCK-8.

\(A_{\text{blank}}\): The absorbance value of the blank group containing medium and CCK-8.

DAPI staining

Cells were treated with adriamycin, GL-V9, and adriamycin combined with GL-V9 for 24 h after seeded onto cover glasses in 6-well plate for about 24 h. After treatments, the
cells were fixed with 4% paraformaldehyde (PFA) for 25 min, washed thrice with cold PBS for 5 min and incubated with 0.2% Triton X-100 for 25 min, then stained with 1 mg·mL⁻¹ diamidino-phenyl-indole (DAPI) for 10 min, and washed thrice with cold PBS for 5 min. The nuclear morphology of cells was examined by fluorescence microscopy (Olympus-slx51; Olympus Corporation, Tokyo, Japan) with a peak-excitation wave length of 340 nm.

**GFP-RFP-LC3 transfection and fluorescence**

The cells were transfected with the plasmid of pCMV GFP-RFP-LC3 using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. At 24 h after transfection, cells were treated with adriamycin, GL-V9, and adriamycin combined with GL-V9 for another 24 h. The fluorescence of GFP-RFP-LC3 was observed. Autophagosomes (yellow dots) and autolysosomes (free red dots) in each cell were counted under the fluorescence microscopy (Leica DM2500). The plasmid of pCMV GFP-RFP-LC3 was kindly provided by Prof. QIANG Lei.

**Cell transfection**

The plasmids of pGMLV-SC5-shNC and pGMLV-SC5-shMapk9 were designed and purchased from Genomeditech (Genomeditech, Shanghai Co., Ltd.). The plasmids of EX-C0742-Lv105 and EX-C0742-Lv105-Mapk9 were bought from GeneCopoeia (Guangzhou GeneCopoeia Co., Ltd.).

To establish JNK2 - knockdown cells and JNK2 - overexpression cells, 293T cells were transfected with lentivirus plasmid together with psPAX2 and pMD2G packing plasmids by X - treme GENE 9 (Roche) for 12 h. Fresh medium was added to 293T cells to replace the previous medium. Then, the supernatants of 293T cells were collected and mixed with fresh medium to infect cells along with 8 μg mL⁻¹ polybrene. Next day, supernatants were replaced with fresh medium containing 1 μg mL⁻¹ puromycin. The puromycin - resistant cells were isolated and used for further experiments.

**Immunofluorescence microscopy**

Cells were seeded onto cover glasses in 6-well plate and fixed by 4% PFA for 30 minutes, followed by permeabilization using 0.3% Triton-X 100 for 30 minutes. Then, the cover glasses were blocked by 3% bovine serum albumin for 1 h and incubated with primary antibody at 4 °C overnight. Next day, cells were incubated with Alexa Fluor conjugated secondary antibodies for 1 h and stained with DAPI for 20 minutes. Confocal microscope (Olympus, Tokyo, JP) was employed to photograph the protein expressions or location in cells.

**Statistical analysis**

Data are presented as mean ± SD from triplicate parallel experiments unless otherwise indicated. Statistical analysis was performed using one-way ANOVA. Least Significant Difference test and Tukey's HSD test were used for the one-way ANOVA analyses.

**Results**

The JNK2-related autophagy increased significantly in adriamycin-resistant cells

We firstly detected the resistance index (RI) of two pairs of adriamycin-resistant cells by the CCK-8 assay and the RI was calculated by GraphPad Prism 6 software (GraphPad Software, San Diego, CA). Results in Figs. 1A, 1B showed that the RI of Hep G2-DOXR was 160.49 and the RI of MCF-7-D0XR was 64.64. Then, we explored the differential proteins between parent cells and adriamycin-resistant cells using western blot assay. We found that the expression of JNK2 was increased significantly in the adriamycin-resistant cells compared with that in parent cells (Fig. 1C). Meanwhile, the expression of autophagy-related proteins such as ATG5, ATG7, Beclin1, p62 and LC3 changed a lot in the adriamycin-resistant cells (Fig. 1C). The results suggested that autophagy increased significantly in the adriamycin-resistant cells than that in the parent cells. These results suggested that the degree of JNK2-related autophagy increased significantly in adriamycin-resistant cells.

The effect of JNK2 on adriamycin sensitivity of Hep G2 cells and Hep G2-DOXR cells

As can be seen from the results of Fig. 1, JNK2 may play an important role in adriamycin resistance of Hep G2 cells. To investigate the effects of JNK2, we overexpressed JNK2 in Hep G2 cells and knocked down JNK2 in Hep G2-DOXR cells. When overexpressing JNK2 in Hep G2 cells, the protective autophagy was activated (Fig. 2A). After incubated with adriamycin for 24 hours, the IC₅₀ of Hep G2-Lv105 cells was 0.69 μmol·L⁻¹ and the IC₅₀ of Hep G2-Lv105-Mapk9 cells was 2.42 μmol·L⁻¹. The adriamycin sensitivity of Hep G2 cells decreased after JNK2 overexpressed (Fig. 2B). When knocking down JNK2 in the Hep G2-DOXR cells, the autophagic flux was blocked (Fig. 2C). The IC₅₀ of Hep G2-DOXR-shNC cells was 52.00 μmol·L⁻¹ and the IC₅₀ of Hep G2-DOXR-shMapk9 cells was 18.86 μmol·L⁻¹ (Table 1). The adriamycin sensitivity of Hep G2-DOXR cells increased after JNK2 knocked down (Fig. 2D). These results indicated that the change of JNK2 expression played an important role in adriamycin resistance.

The autophagic death in Hep G2 cells and Hep G2-DOXR cells

Fig. 1C showed that the autophagy induced by adriamycin-resistant Hep G2 cells was activated significantly and the autophagic flux was blocked. The results suggested that the JNK2-related autophagy increased significantly in the adriamycin-resistant cells.
cin was different between parent cells and adriamycin resist-

ant cells. Then we detected the autophagy-related protein
markers in Hep G2 and Hep G2-DOXR cells after adriamy-
cin was given. Fig. 3 showed that autophagy marker protein
p62 was decreased in Hep G2 cells and LC3-I transforming to
LC3-II was increased after adriamycin treatment. However,
the level of p62 and LC3 didn’t change much in Hep G2-
DOXR cells. The results indicated that the resistance of Hep
G2-DOXR cells to adriamycin was due to the fact that adria-
mycin could not cause autophagic death in the Hep G2-
DOXR cells.

GL-V9 reversed the adriamycin resistance in Hep G2-DOXR
cells

GL-V9 was reported to be a potential candidate of anti-
tumor drug, the chemical structure of it was shown in Fig. 4A.
We detected the sensitivity of Hep G2 and Hep G2-DOXR
cells to GL-V9 by CCK-8 assay (Fig. 4B). We choosed the
dose of 8 μmol·L⁻¹ GL-V9 which had no significant effects
on the survival of the two cells. Then we detected the effects
of 8 μmol·L⁻¹ GL-V9 on the sensitivity of parental Hep G2
and Hep G2-DOXR cells to adriamycin. As shown in Fig. 4C,
when combining with 8 μmol·L⁻¹ GL-V9, the IC50
of Hep G2-DOXR cells was 2.1 times as much as before. The
sensitivity of Hep G2-DOXR cells to adriamycin was in-
creased. Results of Annexin V/DAPI staining also showed
GL-V9 increased the apoptosis-inducing effect of adriamy-

Fig. 1 The JNK2-related autophagy increased significantly in adriamycin-resistant cells. (A) The cell inhibitory rate of adriamy-
cin of Hep G2 cells and Hep G2-DOXR cells was detected by CCK-8 assay. (B) The cell inhibitory rate of adriamycin of MCF-7
cells and MCF-7-DOXR cells was detected by CCK-8 assay (C) The differential proteins between parent cells and adriamycin-
resistant cells were detected by western blot assay. Data were presented as mean ± SD (n = 3). *P < 0.05 , **P < 0.01 vs the group of
Hep G2 or MCF-7
cin in Hep G2-DOXR cells (Fig. 4E). The similar results were observed in DAPI staining (Fig. 4F). In addition, we did the immunofluorescence experiment targeting active caspase-3 and found that when combining with 8 μmol·L⁻¹ GL-V9, the intranuclear distribution of active caspase-3 in Hep G2-DOXR cells increased (Fig. 4G). These results suggested that GL-V9 could reverse the resistance of Hep G2-DOXR cells to adriamycin.

The mechanisms of GL-V9 reversing the resistance of Hep G2-DOXR cells to adriamycin

Regulatory effects between JNK and autophagy have been reported in recent years. In Fig. 5A, when Hep G2 cells treated with adriamycin alone, the autophagic death was induced. However, when treated with low-dose GL-V9 combined with adriamycin, no change of the autophagy and apoptosis happened. However, in Hep G2-DOXR cells, the high ex-
pression of JNK2 activated protective autophagy under non-stress condition. When treated with adriamycin alone, the autophagic death could not be induced. When treated with low-dose GL-V9 combined with adriamycin, the expression of JNK2 was reduced and the autophagic flux was blocked in Hep G2-DOXR cells. Meanwhile, the apoptosis-related pro-

Fig. 4  GL-V9 reversed the adriamycin resistance in Hep G2-DOXR cells. (A) The chemical structure of GL-V9. (B) The cell inhibitory rate of GL-V9 was detected by CCK-8 assay. (C) The cell inhibitory rate of adriamycin on Hep G2 cells combined with GL-V9 or not was detected by CCK-8 assay. (D) The cell inhibitory rate of adriamycin on Hep G2-DOXR cells combined with GL-V9 or not was detected by CCK-8 assay. *P < 0.05, **P < 0.01 vs the group of Hep G2-DOXR. (E) The apoptosis of Hep G2-DOXR cells was detected by Annexin V/DAPI staining. (F) The apoptosis of Hep G2-DOXR cells was detected by DAPI staining and observed by fluorescence microscopy (original magnification, ×200). (G) The apoptosis of Hep G2-DOXR cells was detected by the immunofluorescence experiment targeting active caspase-3 (original magnification, ×200)
Fig. 5  The mechanisms of GL-V9 reversing the resistance of Hep G2-DOXR cells to adriamycin. (A) The expression of autophagy-related proteins and apoptosis-related proteins was detected by western blot assay. Data were presented as mean ± SD (n = 3). *P < 0.05, **P < 0.01 vs the DOX (5 μmol·L−1) group. (B) The changes of autophagy influx in Hep G2 and Hep G2-DOXR cells were observed by fluorescence microscopy (original magnification, × 400). (C) The expression of autophagy-related proteins and apoptosis-related proteins in Hep G2-DOXR-shMapk9 was detected by western blot assay. Data were presented as mean ± SD (n = 3)
teins such as Bcl-2, BAX, PARP1 and Active caspase-3 were changed significantly. The process of autophagy is dynamic. The formation of autophagosome, the transport of autophagic substrate to lysosome and the degradation of lysosome are called autophagic flux. GFP-RFP-LC3 double fluorescence autophagy indicator system is used to label and track the changes of LC3 and autophagy influx. Then we observed the change of autophagic flux of Hep G2 and Hep G2-DOXR cells by GFP-RFP-LC3 double fluorescence (Fig. 5B), we found that when Hep G2 cells were treated with adriamycin alone, the autolysosomes formed and the fluorescence intensity of GFP was decreased. The autophagic flux was normal. When Hep G2 cells were treated with low-dose GL-V9 combined with adriamycin, the autophagic flux almost did not change. However, when Hep G2-DOXR cells were treated with low-dose GL-V9 combined with adriamycin, the autophagosomes formed and the intensity of GFP did not change. The autophagic flux was blocked.

To verify the role of JNK2 in the process of GL-V9 reversing adriamycin resistance, JNK2-knockdown cells were used. When JNK2 was knocked down (Fig. 5C), the autophagy-related proteins such as p62 and LC3 had no difference in the adriamycin-alone group and adriamycin combined with GL-V9 group. There was no change in apoptosis-related proteins such as Bcl2, Bax, PARP1, and Active caspase-3 as well. It indicated that GL-V9 could not reverse adriamycin resistance of Hep G2-DOXR cells any more when JNK2 was knocked down.

All the results suggested that GL-V9 reversed adriamycin resistance of Hep G2-DOXR cells by blocking JNK2-related protective autophagy.

**Discussion**

The aim of the study was to explore the effects of JNK2-related autophagy on adriamycin resistance and the reversal effects of the new synthesized flavonoid GL-V9 on adriamycin resistance. The findings of the study suggested that adriamycin combined with GL-V9 could be a promising treatment in surviving HCC cells when adriamycin resistance existed.

As an available chemotherapeutic drug, adriamycin is an anti-tumor antibiotic, a potent cytotoxic drug that suppresses the synthesis of RNA and DNA. These findings are of importance for the understanding of HCC recurrence observed subsequent to transarterial chemoembolization (TACE) [23]. There have been many studies that have shown a strong relationship between the level of autophagy and chemotherapy resistance. In our study, the transformation of autophagy marker protein LC3 from LC3-I to LC3-II increased and the expression of p62 decreased significantly in adriamycin resistant cells. All the results showed that autophagy levels were significantly elevated in adriamycin resistance. It has been reported that the downregulation of the JNK signaling pathway can down-regulate the level of MDR and reverse the multidrug resistance [28]. Whether the change of JNK2 expression can affect the sensitivity of adriamycin has not been studied. We found that JNK2 could obviously affect the sensitivity of adriamycin in Hep G2 cells and Hep G2-DOXR cells.

By detecting the cell inhibitory rate of adriamycin of Hep G2-DOXR cells, we found that GL-V9 combined with adriamycin was very effective. However, GL-V9 could not increase the sensitivity of Hep G2 cells to adriamycin. The difference between the effect of GL-V9 on the sensitivity to adriamycin of parent cells and adriamycin-resistant cells is due to the different expression of JNK2 in the two kinds of cells. We also observed that GL-V9 could block protective autophagy by down-regulating the expression of JNK2 which finally reversed adriamycin resistance of HCC. Thus, GL-V9 could be used as a specific blocker of JNK2-related autophagy in the future.

**References**


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