

A novel curcumin analogue is a potent chemotherapy candidate for human hepatocellular carcinoma

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Received May 16, 2015; Accepted August 10, 2016

DOI: 10.3892/ol.2016.5126

Abstract. Curcumin (CUR) has been demonstrated to protect against carcinogenesis and to prevent tumor development in cancer; however, the clinical application of CUR is limited by its instability and poor metabolic properties. The present study offers an strategy for a novel CUR analogue, (1E,4E)-1,5-bis(2-bromophenyl)penta-1,4-dien-3-one (GL63), to be used as a potential therapeutic agent for hepatocellular carcinoma (HCC) *in vitro* and *in vivo*. The current study demonstrated that GL63 exhibited more potent inhibition of proliferation of HCC cells than CUR. GL63 induced G0/G1 phase cell cycle arrest and apoptosis in SK-HEP-1 cells in a dose-dependent manner, and was more potent than CUR, according to the flow cytometry data. The present study demonstrated for the first time that the inhibition of the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway by GL63 resulted in a protective effect against HCC cell growth. GL63 was more effective than CUR in regulating STAT3 downstream targets, which contributed to the suppression of cell proliferation and the induction of cell apoptosis. In addition, the effects of GL63 were tested in a model of N-nitrosodiethylamine (DEN)-induced HCC in Wistar rats. Although macroscopic and microscopic features suggested that both GL63 and CUR were effective in inhibiting DEN-induced hepatocarcinogenesis, GL63 exerted a stronger effect than CUR. Immunohistochemical analysis for proliferating cell nuclear antigen demonstrated significant differences among the DEN-bearing non-treated, DEN-bearing GL63-treated and DEN-bearing, CUR-treated groups ($P=0.039$). It was concluded that GL63 was a potent agent able to suppress the proliferation of HCC cells by inhibition of the JAK2/STAT3 signaling pathway, with more favorable pharmacological activity than CUR, and may be a

more potent compound for the prevention of DEN-induced hepatocarcinogenesis in rats than CUR.

Introduction

Hepatocarcinoma is one of the major malignant tumors in humans, with ~600,000 newly diagnosed cases, and leading to >250,000 mortalities annually (1,2). Surgical resection, in the form of partial hepatectomy or total hepatectomy, followed by liver transplantation, may provide an occasional incidence of cure (3). However, this can be performed only in selected patients whose tumors are small and located away from major vessels and have not metastasized to extrahepatic organs (4). No chemotherapy agent has better benefit than surgery in controlled clinical trials due to different reasons, including drug resistance and toxicity to normal cells (5).

The development of innovative novel therapeutics for the management of hepatocarcinoma is particularly urgent, and this development is the long-term objective of the current study. Curcumin (CUR) has been reported to interrupt the cell cycle, exert cytotoxic effects, and participate in antiproliferation and induction of apoptosis in numerous hepatocarcinoma cell lines *in vitro* (6). A considerable number of reports have also described the anticancer effects of CUR on hepatocellular carcinoma (HCC) *in vivo* (7). Although CUR is remarkably non toxic and has promising anticancer activities, preclinical and clinical studies indicate that its poor bioavailability and pharmacokinetic profile due to its instability under physiological conditions have limited its application in anticancer therapies (8-10). Thus, the development of synthetic structural analogues of CUR is one approach for overcoming the poor bioavailability of CUR while retaining or further enhancing its drug-like effects (11,12). The present authors have designed and synthesized a series of mono-carbonyl analogues of CUR by deleting the reactive β -diketone moiety (13-15). Although previous studies suggest that the presence of the β -diketone moiety may be necessary for the biological activities of CUR, a number of studies from several independent groups demonstrated that certain CUR analogues containing a 5-carbon enone spacer without β -diketone either retained or increased the growth-suppressive activities of CUR against several cancer cells (16,17). Those studies indicate that certain mono-carbonyl analogues not only have enhanced stability and antitumor activities *in vitro*, but also

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Key words: hepatocellular carcinoma, curcumin, analogue, diethylnitrosamine, signaling pathway

have better pharmacokinetic profiles *in vivo* than CUR (18). One such compound, (1E,4E)-1,5-bis(2-bromophenyl)penta-1,4-dien-3-one (GL63) was synthesized as part of a series of novel CUR analogues (19).

Upon oral administration at a dose of 500 mg/kg GL63 and CUR to rats, the concentration of each compound in plasma was measured by high performance liquid chromatography (20). GL63 was observed to possess a plasma concentration ~44-fold higher than that of CUR (area under the curve) and a peak blood concentration ~45-fold higher than that of CUR (20). Furthermore, there was an apparent decrease in clearance with GL63 (38.98 l/kg/h) compared with CUR (835.20 l/kg/h) (20). In summary, these pharmacokinetics data indicate that the deletion of the β -diketone moiety significantly decreases the degree and speed of metabolism of curcuminoids, and that GL63 possesses a much better pharmacokinetic profile than CUR (20). Previous studies demonstrated that the cytotoxic effect of GL63 against HepG2 human hepatocellular carcinoma and CNE2 nasopharyngeal carcinoma cells was due to the induction of cell cycle arrest and subsequent apoptosis by the endoplasmic reticulum stress pathway (21,22). Xiao *et al* noticed that treatment of H460 human lung epithelial cancer cells with GL63 facilitated the degradation of cyclooxygenase-2 messenger (m) RNA, as evidenced by mRNA degradation assay (19). Xiao *et al* also observed that neither GL63 nor CUR induced apoptosis in normal liver cells, which indicated that GL63 had no significant toxicity, similar to CUR (21). These results suggested that the novel CUR-related compound GL63 is a potent antitumor agent. To date, although GL63 has been used for various different medical purposes *in vitro* (21,22), the underlying cellular and molecular mechanisms by which GL63 suppresses hepatocarcinoma cell growth are unknown. Whether GL63 has potential as a novel therapeutic agent for hepatocarcinoma to the same extent than CUR remains to be investigated. In the present study, the inhibitory efficacy of GL63 was evaluated on hepatocarcinoma *in vitro* and *in vivo*, and it was assessed whether GL63 was more potent than CUR in inhibiting the growth of liver cancer cell lines (SK-HEP-1) and of hepatocarcinoma induced by N-nitrosodiethylamin (DEN) in a Wistar rat model.

Materials and methods

Cell lines and reagents. GL63 was synthesized as reported by Liang *et al* (20). The SK-HEP-1 cell line was purchased from the Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 medium (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck Millipore) and antibiotics (100 U/ml penicillin and 100 μ g/ml streptomycin) in cell culture incubators set at 37°C and aired with 5% CO₂.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. The MTT assay was used to evaluate cell viability. Briefly, cells were seeded onto 96-well plates, and allowed to adhere and grow in 10% FBS-containing RPMI-1640 medium for 24 h. GL63 (Hebei University of Science and Technology, Shijiazhuang, China) and CUR (Hebei University of Science and Technology) were

dissolved in dimethyl sulfoxide (DMSO). The cells were then treated with various concentrations of GL63 and CUR (0, 10, 20 and 40 μ M) for 24 h. MTT (20 μ l at 5 mg/ml) was added to each well, and incubation was conducted for 3.5 h. MTT was aspirated, and 100 μ l DMSO was added to each well. Next, the absorbance at 570 nm was read in a plate reader. The half-maximal inhibitory concentration (IC₅₀) was used as the concentration of drug required to obtain 50% of maximal inhibition in cell viability. Each treatment was performed in triplicate. The mean of three values was determined, and the results were expressed as a percentage of the control. Cell viability was expressed as the percentage of the absorbance in the treated wells relative to that of the untreated (control) wells. Three independent experiments were performed.

Cell cycle analysis. SK-HEP-1 cells were seeded in 6-well plates at a concentration of 5x10⁵ cells/well. Following treatment with various concentrations of GL63 and CUR (0, 10, 20 and 40 μ M) for 24 h, the cells were harvested and washed with phosphate-buffered saline (PBS), and then resuspended in 70% ethanol at -20°C overnight. Next, the cells were washed twice with PBS and 10 mg/ml RNase A was added. Propidium iodide (PI) was added to the tubes at a final concentration of 0.05 mg/ml and incubated at 4°C for 30 min in the dark. Cell cycle analysis was conducted immediately upon staining using an EPICS Elite ESP flow cytometer (Beckman Coulter, Inc., Brea, CA, USA) and WinMDI 2.9 software (Beckman Coulter, Inc.).

Flow cytometric analysis. SK-HEP-1 cells were seeded in 6-well plates at a density of 5x10⁵ cells/well for 24 h at 37°C, incubated with 20 μ M Z-DEVD-FMK (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) or vehicle for 1 h, followed by treatment with various concentrations of GL63 and CUR (0, 10, 20 and 40 μ M) for 24 h. All cells were collected and washed twice with cold PBS. Next, the cells were collected and resuspended in 100 μ l binding buffer containing annexin V and PI. The mixed solution was gently vortexed and incubated in the dark at room temperature (25°C) for 15 min. Quantification of annexin V and PI binding was performed with a FACScan™ (BD Biosciences, Franklin Lakes, NJ, USA).

Western blotting. Following treatment with 0, 10, 20 and 40 μ M CUR and GL63 for 24 h, the culture medium was collected, and the cells were washed with ice-cold PBS. Cells were lysed with lysis buffer (Beijing Biosynthesis Biotechnology Co. Ltd., Beijing, China) for 30 min at 40°C, followed by centrifugation at 20,000 x g for 10 min at 4°C. The supernatants were collected and the protein concentrations were determined using a BCA-100 Protein Quantitative Analysis kit (Beijing Biosynthesis Biotechnology Co. Ltd.). Proteins were then subjected to electrophoresis on 10% SDS-PAGE gels and transferred to a polyvinylidene difluoride blotting membrane for 1 h at room temperature. Membranes were blocked with 5% skim milk at room temperature for 1 h, followed by incubation with polyclonal rabbit phosphorylated (p)-Janus kinase 2 (JAK2; cat. no. sc-16566; 1:1,000), JAK2 (cat. no. sc-278; 1:1,000), p-signal transducer and activator of transcription 3 (STAT3; cat. no. sc-135649; 1:1,000), STAT3 (cat. no. sc-7179; 1:1,000), cytochrome c (Cyt-c; cat. no. sc-33174; 1:1,000), caspase-9 (cat. no. sc-7885; 1:1,000),

caspase-3 (cat. no. sc-98785; 1:1,000), poly(ADP-ribose) polymerase (PARP; cat. no. sc-133888; 1:1,000), B-cell lymphoma (Bcl)-2 (cat. no. sc-783; 1:1,000), Bcl- extra large (xL) (cat. no. sc-7195; 1:1,000) and Bcl-2 associated X protein (Bax; cat. no. sc-493; 1:1,000) rabbit polyclonal antibody (Santa Cruz Biotechnology Inc.) primary antibodies at 4°C overnight. Following three washes with TBST, the membranes were incubated with horseradish peroxidase-conjugated polyclonal goat anti-rabbit IgG secondary antibody (cat. no. sc-2004; 1:10,000; Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Chemiluminescence detection was performed using an ECL Western Blotting Detection kit (GE Healthcare Life Sciences, Chalfont, UK). The quantities of the proteins were analyzed using ImageJ 1.48 analysis software and normalized to their respective control.

Tumorigenicity assays in Wistar rats. A total of 105 healthy 4-week-old male Wistar rats (100-110 g) were provided by the Animal Center of Beijing Medical University (Beijing, China). All animals received humane care, and protocols were approved by the Hebei Medical University Animal Ethics Committee (Shijiazhuang, China). The animals were housed in a polypropylene cage at temperature of 22±2°C and 50-60% relative humidity, with a 12 h light/dark cycle for 1 week prior to and during the experiment. Rats were allowed to access standard pelleted rat chow and water. Group 1 (control non-treated group; n=10) was considered as the normal control group. GL63 in group 2 (control GL63-treated group; n=10) was administered intraperitoneally (80 mg/kg) twice a week for 12 weeks. CUR in group 3 (control CUR-treated group; n=10) was administered intraperitoneally (80 mg/kg) twice a week for 12 weeks. The rats in group 4 (DEN-bearing non-treated group; n=25) were administered an intraperitoneal injection of DEN (50 mg/kg) twice a week for 12 weeks. The rats in group 5 (DEN-bearing GL63-treated group; n=25) were administered both DEN as in group 4 and GL63 as in group 2. The rats in group 6 (DEN-bearing, CUR-treated group; n=25) were administered both DEN as in group 4 and CUR as in group 3. Body weight was recorded at the end of every week for 24 weeks. The experiment was terminated at the end of 24 weeks, and all the surviving rats were anaesthetized and sacrificed at the end of the experiment, following animal ethics guidelines (23).

Cytomorphological evaluation and immunohistochemistry. Autopsy specimens were obtained from the liver of rats in all groups. All the liver tissues were fixed routinely and embedded in paraffin to prepare 4-μm sections for hematoxylin and eosin staining. The diagnosis and grade of hepatocarcinoma was established based on the morphological findings identified on the cell block sections using the World Health Organization criteria (24). The expression strength of proliferating cell nuclear antigen (PCNA; polyclonal rabbit antibody; cat. no. sc-7909; 1:100; Santa Cruz Biotechnology, Inc.) was based on the staining intensity (0, negative; 1, weak; 2, intermediate; and 3, strong) and the percentage of positive cells (0, 0% positive cells; 1, ≤25% positive cells; 2, 26-50% positive cells; and 3, >50% positive cells) identified following incubation with the PCNA antibody for 1 h at room temperature. The two scores were added, with a maximum score of 6. A score of

>2 represented a positive immunohistochemical identification of the marker.

Statistical analysis. All statistical analysis was performed using SPSS 13.0 statistical software (SPSS, Inc., Chicago, IL, USA). All qualitative variables are expressed as frequencies and percentages. χ^2 test and Fisher's exact test were used to determine associations between categorical variables. All quantitative data are expressed as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

GL63 inhibits cell viability and proliferation more potently than CUR. The cytotoxicity that GL63 and CUR effected on SK-HEP-1 cells was assessed by MTT assay. When cultured with various concentrations of GL63 (0, 10, 20 and 40 μM), the proliferation of SK-HEP-1 cells was significantly suppressed in a dose-dependent manner, with an IC₅₀ value of 14.8±1.4 μM, which indicated that GL63 was substantially more potent than CUR (Fig. 1). Therefore, the results indicated that GL63 could efficiently inhibit the growth of SK-HEP-1 cells to a better extent than CUR.

GL63 is more potent than CUR in inducing cell cycle arrest. In order to gain further information about the mechanism of the cell-growth inhibitory effect of GL63, the cell cycle distribution of SK-HEP-1 cells was assessed by flow cytometry following 24-h treatment with various concentrations of GL63 and CUR (0, 10, 20 and 40 μM). As indicated in Fig. 2, GL63 increased the number of SK-HEP-1 cells in the G0/G1 phase of the cell cycle, whereas CUR caused a weaker change in the cell cycle distribution. The average histogram plot of cell-cycle analysis also indicated that GL63 treatment induced a dose-dependent accumulation of SK-HEP-1 cells in G0/G1 phase. These results suggested that GL63 caused a cell-cycle arrest in G0/G1 phase.

GL63 promotes cell apoptosis more potently than CUR. To further investigate the underlying mechanism of decreased cell proliferation, the apoptosis of SK-HEP-1 cells induced by GL63 was examined using annexin V/PI assay (Fig. 3A and B). The apoptosis of SK-HEP-1 cells (including early and late apoptotic cell death) increased in a dose-dependent manner following treatment with GL63 for 24 h. Furthermore, GL63 significantly induced apoptosis compared with CUR at the same concentrations in SK-HEP-1 cells. Bcl and caspase family proteins are widely accepted to mediate the apoptotic pathway (25). Mitochondrial apoptotic pathway-related proteins (caspase-3, caspase-9, Cyt-c and PARP) were upregulated by GL63 treatment, suggesting that this apoptotic pathway was activated (Fig. 3C and D). It was observed an increased activation of caspase-3 in SK-HEP-1 cells treated with increasing concentrations of GL63, indicating that GL63-induced apoptosis was dependent upon caspase-3 activity. A similar tendency was observed for caspase-9, Cyt-c and PARP. Since caspase-9 activation involves mitochondria permeability changes and the release of Cyt-c (21), a significant increase in released Cyt-c was detected by GL63 treatment

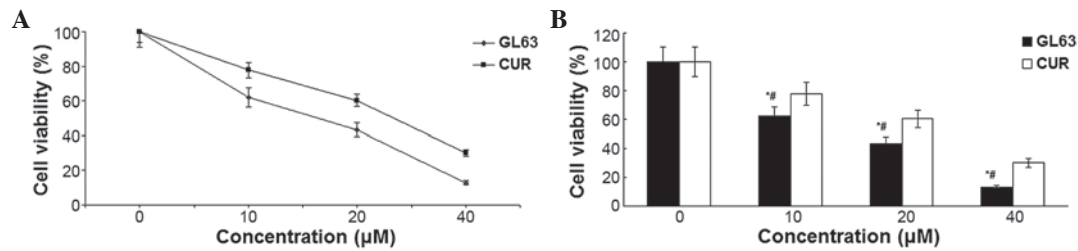


Figure 1. Analysis of cell viability. (A) Effect of GL63 and CUR on the cell viability of SK-HEP-1 cells following treatment with 0–40 μ M GL63 and CUR for 24 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. (B) Bar diagram of cell viability analysis from three independent treatments. Data are presented as the mean \pm standard deviation. * P <0.05 vs. dimethyl sulfoxide control. # P <0.05 vs. CUR group at the same concentration. CUR, curcumin; GL63, (1E,4E)-1,5-bis(2-bromophenyl)penta-1,4-dien-3-one.

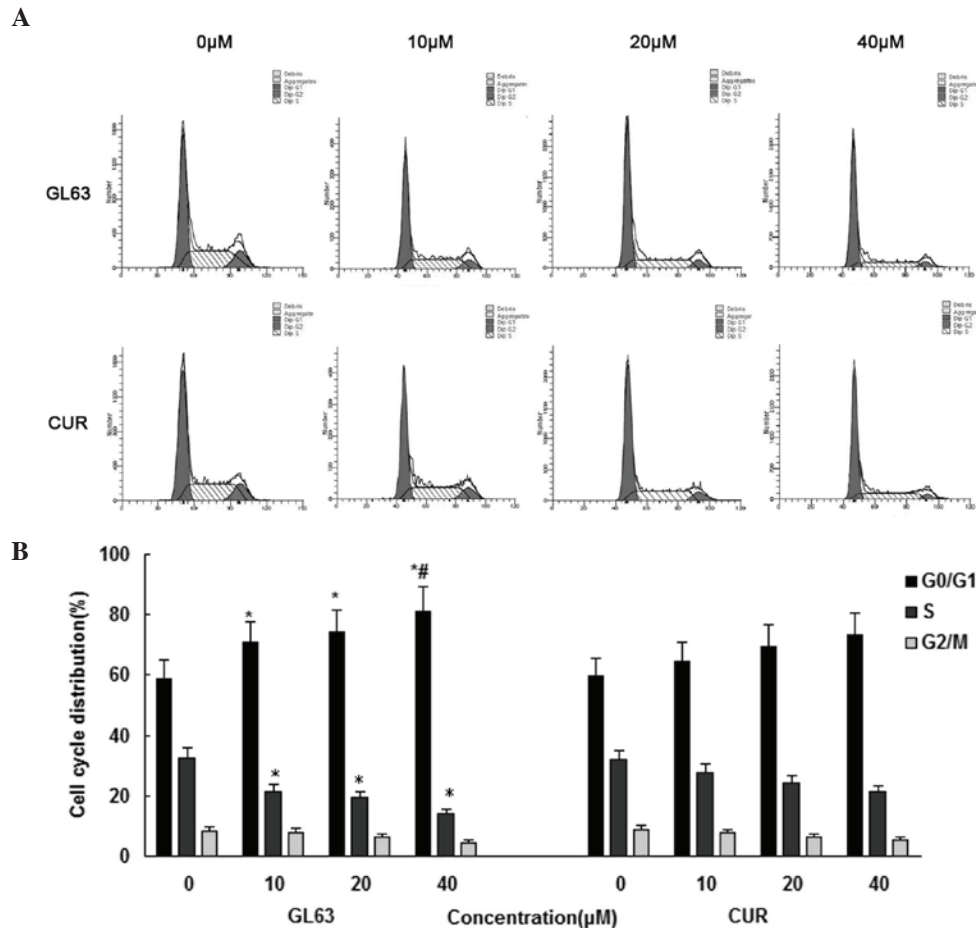


Figure 2. Effects of GL63 and CUR on cell cycle distribution in SK-HEP-1 cells. (A) SK-HEP-1 cells were treated with GL63 and CUR (0, 10, 20 and 40 μ M) for 24 h, stained with propidium iodide and analyzed with a BD FACScan™ flow cytometer. (B) Bar graph of cell cycle distribution representing the mean \pm standard deviation from three independent experiments. * P <0.05 vs. dimethyl sulfoxide control. # P <0.05 vs. CUR group at the same concentration. CUR, curcumin; GL63, (1E,4E)-1,5-bis(2-bromophenyl)penta-1,4-dien-3-one.

at various doses. Cells were pretreated with the caspase-3 inhibitor Z-DEVD-FMK prior to and throughout treatment with GL63, and it was observed that Z-DEVD-FMK almost completely inhibited GL63-induced apoptosis in SK-HEP-1 cells, indicating that GL63-induced apoptosis was dependent upon caspase-3 activity (Fig. 3E). Notably, CUR did not induce caspase activation markedly, compared with GL63. These *in vitro* results support a mitochondria-dependent caspase activation to mediate GL63-induced apoptosis. The details of the mechanisms by which GL63 affects the mitochondria to initiate apoptosis signaling pathways will require further investigation.

GL63 potently suppresses the JAK2/STAT3 signaling pathway and regulates the expression of its downstream targets *in vitro*. To characterize the molecular mechanism by which the JAK2/STAT3 signaling pathway alters GL63-induced apoptosis in SK-HEP-1 cells, the expression of proteins in response to GL63 was examined by immunoblotting. The results indicated that the levels of JAK2 and STAT3 were not altered, while the levels of p-JAK2 and p-STAT3 were markedly and concentration-dependently reduced following treatment with GL63 and CUR in SK-HEP-1 cells. GL63 significantly reduced the level of p-JAK2 and p-STAT3 at various concentrations, while CUR

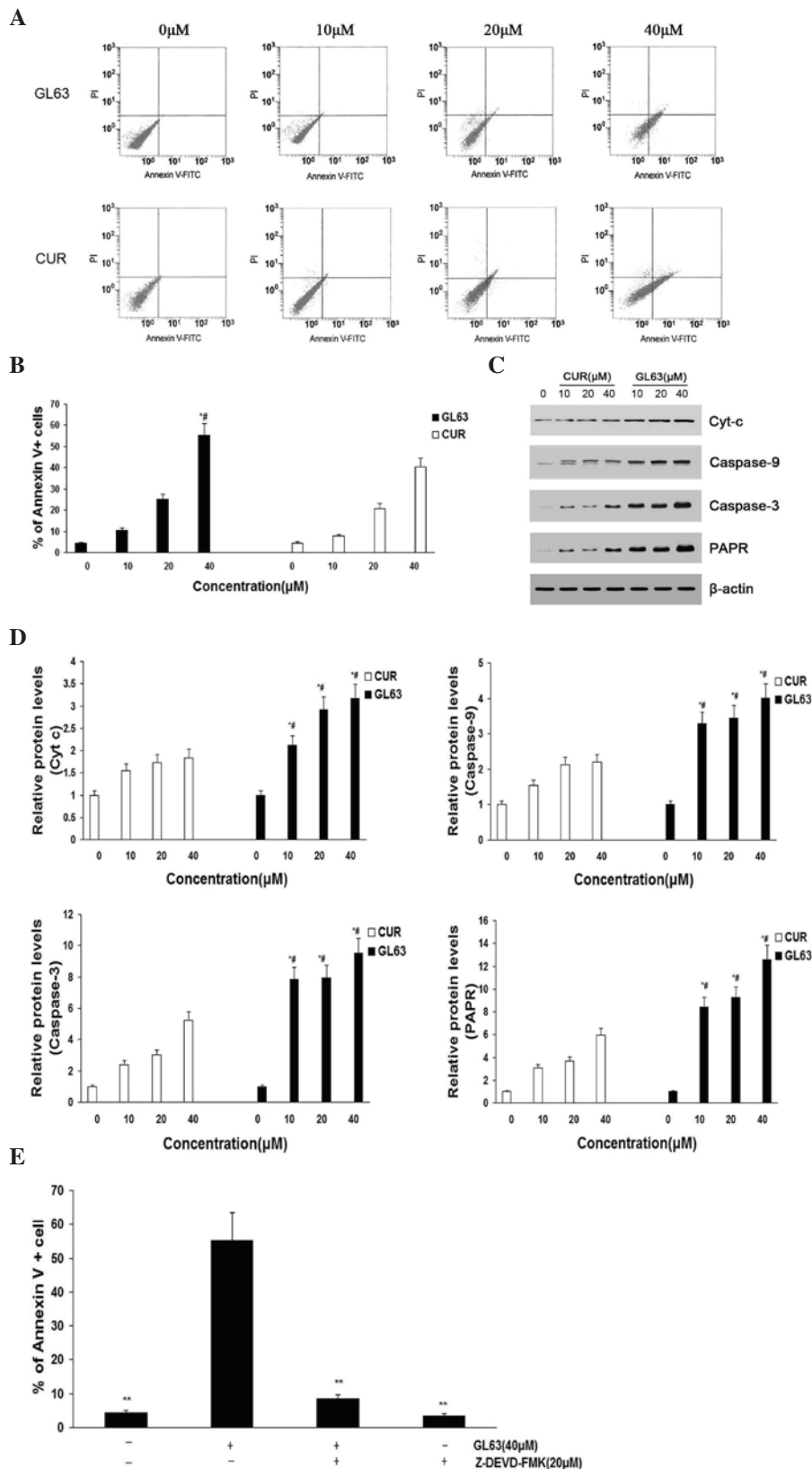


Figure 3. Apoptosis rates of SK-HEP-1 cells subjected to GL63 and CUR treatment, as detected by flow cytometry and protein expression analysis. SK-HEP-1 cells were treated with GL63 and CUR (0, 10, 20 and 40 μ M) for 24 h. (A) Representative diagrams of annexin V-fluorescein isothiocyanate/propidium iodide staining upon treatment with different concentrations of GL63 and CUR. (B) Bar diagram of apoptotic cell rate from three FACS analyses from three separate treatments. (C) Caspase-9, caspase-3, Cyt-c and PARP levels were determined in SK-HEP-1 cells treated with GL63 and CUR at various concentrations for 24 h. β -actin was used as a protein loading control. (D) Bar diagram represents the expression levels of caspase-9, caspase-3, Cyt-c and PARP, which displayed a concentration-dependent increase upon treatment with GL63 or CUR. (E) Z-DEVD-FMK inhibits GL63-induced apoptosis. SK-HEP-1 cells were incubated with Z-DEVD-FMK (20 μ M) or vehicle for 1 h, followed by treatment with or without GL63 (40 μ M) for another 24 h, and then apoptosis was evaluated by FACS (** P <0.01 vs. GL63 alone treatment). All data were expressed as the mean \pm standard deviation of ≥ 3 separate experiments. * P <0.05 vs. dimethyl sulfoxide control. ** P <0.05 vs. CUR group at the same concentration. CUR, curcumin; FITC, fluorescein isothiocyanate; PI, propidium iodide; Cyt-c, cytochrome c; PARP, poly(ADP-ribose) polymerase; FACS, fluorescence-activated cell sorting; GL63, (1E,4E)-1,5-bis(2-bromophenyl)penta-1,4-dien-3-one.

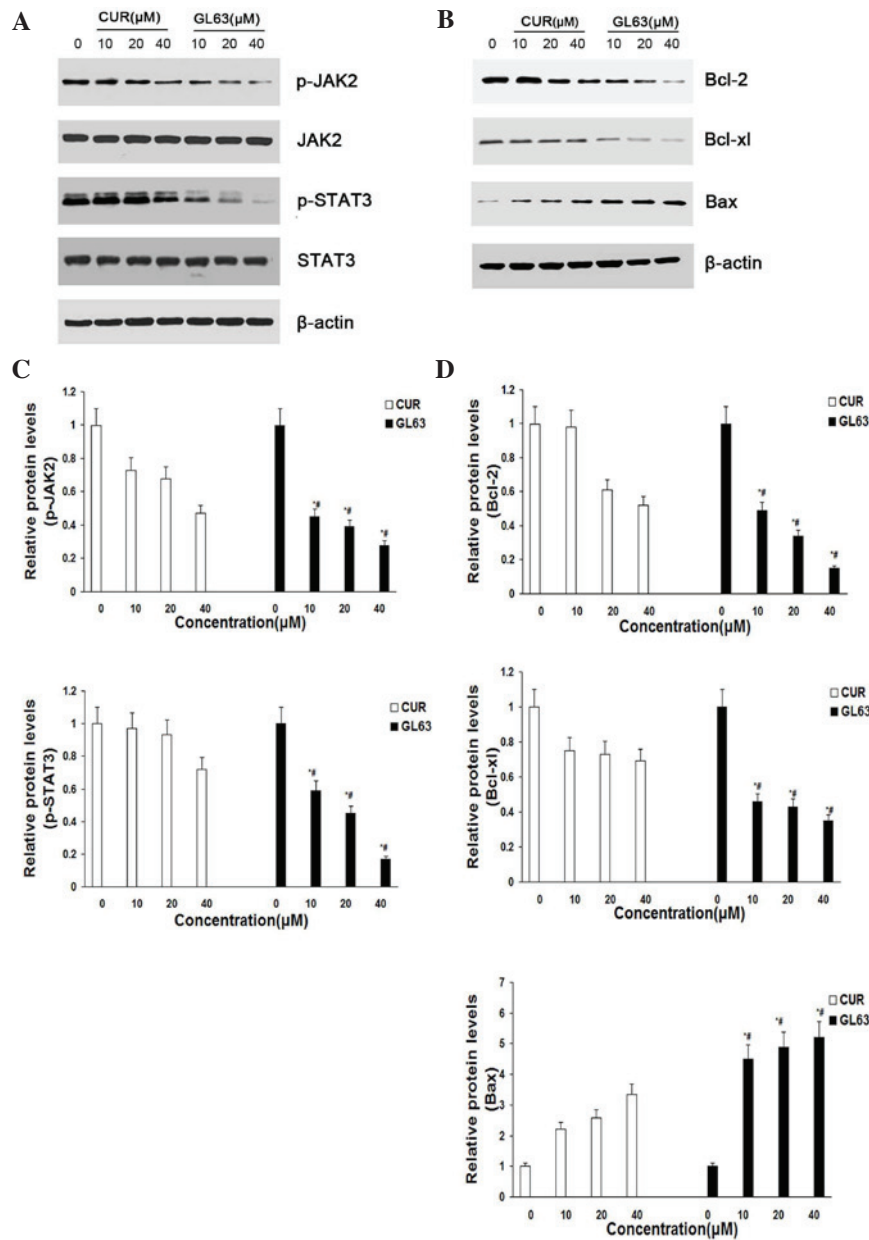


Figure 4. GL63 and CUR both reduce cell viability in human SK-HEP-1 cells by inhibiting the JAK2/STAT3 signaling pathway and its downstream gene expression. (A) SK-HEP-1 cells were treated with the indicated concentrations of GL63 and CUR for 24 h prior to be subjected to western blot analysis with anti-p-JAK2, anti-JAK2, anti-p-STAT3 and anti-STAT3 antibodies. (B) Bar diagram of the effects of the different concentrations of GL63 and CUR on p-JAK2 and p-STAT3 from three separate treatments for 24 h. (C) Representative immunoblots of Bcl-2, Bcl-xl and Bax from the nuclear extracts of SK-HEP-1 cells treated with GL63 and CUR (0, 10, 20 and 40 μ M) for 24 h. β -actin served as a loading control. (D) Bar diagram of the effects of the different concentrations of GL63 and CUR on STAT3 downstream gene expression from three separate treatments. Data are presented as the mean \pm standard deviation. * $P < 0.05$ vs. dimethyl sulfoxide control. # $P < 0.05$ vs. CUR group at the same concentration. CUR, curcumin; JAK2, Janus kinase 2; STAT3, signal transducer and activator of transcription 3; p-, phosphorylated; Bcl-2, B-cell lymphoma-2; Bcl-XL, B-cell lymphoma-extra large; Bax, Bcl-2 associated X protein; GL63, (1E,4E)-1,5-bis(2-bromophenyl)penta-1,4-dien-3-one.

only slightly reduced p-JAK2 and p-STAT3 expression (Fig. 4A and C). To further investigate whether GL63 could affect STAT3 downstream genes, western blot analysis was used to examine the expression of Bcl-2, Bcl-xl and Bax. The results indicated that cells treated with GL63 exhibited a strong reduction in the protein expression levels of Bcl-2 and Bcl-xl and a concomitant increase in Bax expression in a concentration-dependent manner, compared with CUR (Fig. 4B and D).

Liver tumor formation. Table I presents the results of survival, tumor incidence and number of nodules per nodule-bearing

liver in rats. The hepatocarcinogenic rats were infiltrated with a large number of nodules, thus making the precise assessment of tumor mass impossible. The literature reports that, in previous studies, nodules measuring >6 mm in diameter at week 24 were always hepatocarcinoma (26); thus, in the present study, those nodules were quantified according to this standard when detected at the surface of the liver. As indicated in Table I, no tumors were observed in the untreated control group (group 1) or in the GL63 or CUR control groups (groups 2 and 3). A significant reduction in tumor incidence was observed in the GL63 and CUR-treated groups (group 5 and 6) compared with

Table I. Effect of GL63 and CUR on the survival and development of nodules on DEN-induced hepatocarcinogenesis in rats.

Group	Treatment	Survival, %	Tumor incidence, %	Average count of nodules/ nodules bearing liver
1	Control	100.0 (10/10)	-	-
2	GL63 alone	100.0 (10/10)	-	-
3	CUR alone	100.0 (10/10)	-	-
4	DEN alone	52.0 (13/25) ^a	100.0 (25/25) ^a	17.53±1.29 ^a
5	DEN + GL63	92.0 (23/25) ^c	20.0 (5/25) ^c	10.75±1.72 ^c
6	DEN + CUR	80.0 (20/25) ^b	36.0 (9/25) ^c	13.14±1.64 ^c

Results are expressed as the mean ± standard deviation. ^aP<0.01 vs. control group. ^bP<0.05, ^cP<0.01 vs. DEN alone group. DEN, N-nitrosodiethylamine; CUR, curcumin; GL63, (1E,4E)-1,5-bis(2-bromophenyl)penta-1,4-dien-3-one.

Table II. Effect of GL63 and CUR on body, liver and relative liver weights on DEN-induced hepatocarcinogenesis in rats.

Group	Treatment	Gain in body weight, g	Final body weight, g	Liver weight, g	Relative liver weight, g
1	Control	267.1±4.7	371.9±5.5	11.38±0.12	3.06±0.04
2	GL63 alone	265.0±8.7	371.2±9.5	11.47±0.12	3.10±0.08
3	CUR alone	263.9±4.6	368.9±4.8	11.48±0.11	3.11±0.06
4	DEN alone	196.2±10.8 ^a	302.0±11.1 ^a	14.23±0.27 ^a	4.73±0.16 ^a
5	DEN + GL63	245.5±9.7 ^c	350.2±9.7 ^c	12.01±0.20 ^b	3.44±0.10 ^c
6	DEN + CUR	243.4±8.1 ^c	347.5±8.2 ^c	11.95±0.30 ^b	3.44±0.09 ^c

Results are expressed as the mean ± standard deviation. ^aP<0.01 vs. to control group. ^bP<0.05, ^cP<0.01 vs. DEN alone group. DEN, N-nitrosodiethylamine; CUR, curcumin; GL63, (1E,4E)-1,5-bis(2-bromophenyl)penta-1,4-dien-3-one.

the DEN-bearing non-treated group (group 4). Groups 5 and 6 exhibited a significant decrease in the number of nodules as compared with group 4. Furthermore, GL63 appeared to be a more potent compound than CUR, with regard to decreasing tumor incidence and nodules numbers in rats. Table II presents data regarding body weight, absolute liver weight and relative liver weight of the rats from each control and experimental group. The body weights were significantly decreased in the DEN-bearing non-treated group compared with the control groups (P<0.001). GL63 and CUR treatment of rats with DEN significantly improved their body weight (P<0.001), compared with rats in group 4. Group 4 exhibited an increased liver/body weight ratio compared with group 1. There was an appreciable decrease in the absolute and relative liver weight in groups 5 and 6 compared with rats administered DEN alone, particularly in group 5.

Histopathological observations. The histology of the liver tissue was examined under a light microscope and represented in (Fig. 5). The livers from the control non-treated group (group 1) exhibited a normal architecture and cytoplasm of hepatic cells, displaying granulated cytoplasm, a central vein, small uniform nuclei and nucleoli. Although liver tissues from rats treated with GL63 only (group 2) displayed no significant differences compared with those of the rats in the control non-treated group (P=0.267), they exhibited minor sinusoidal congestions. Compared with

group 2, the liver tissues from rats treated with CUR only (group 3) also had a normal lobular organization, represented by a central vein, hepatic cords and sinusoids, but displayed moderate-to-severe sinusoidal and venous congestions. DEN treatment alone (group 4) resulted in the loss of the normal architecture, with the tissues displaying instead a granular cytoplasm with large hyperchromatic nuclei. Although the DEN-bearing GL63-treated group (group 5) lost the normal organization of hepatic lobules, it exhibited cancerous foci with patchy necrosis compared with group 4. The tissues of rats in the DEN-bearing CUR-treated group (group 6) exhibited disorganized cells bordering wide sinusoids, but displayed moderately malignant features such as focal necrosis and a low mitotic count.

Immunohistochemical examinations. PCNA was expressed in the nuclei of hepatic cells. The number of PCNA-positive tumor cells was significantly lower in the DEN-bearing treated with GL63 or CUR group than in the DEN-bearing non-treated group, particularly in group 5 (all P<0.05). Photomicrographs of PCNA immunohistochemical sections of various groups are represented in Fig. 6.

Discussion

The liver is the key organ of the metabolic, secretory and excretory functions in the body, and its disorders are numerous

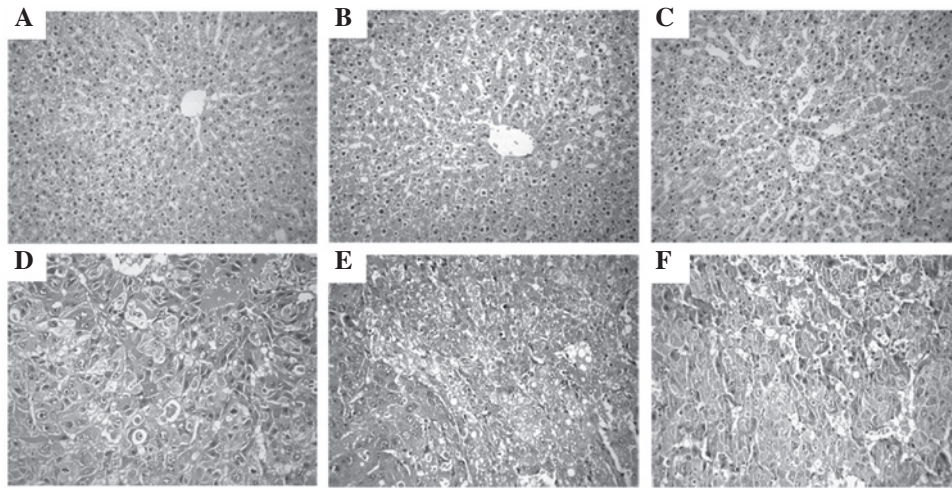


Figure 5. Observation of histopathology in liver. (A) Hepatic tissues from rats in the control non-treated group revealed a normal architecture. (B) Hepatic tissues from rats treated with GL63 only displayed mild sinusoidal and venous congestion. (C) Hepatic tissues from rats treated with CUR displayed more venous and sinusoidal congestion than those of panel B. (D) Cancerous livers from rats treated with DEN only exhibited loss of architecture with cancerous foci. (E) Liver tissues from rats in the DEN-bearing GL63-treated group exhibited a normal architecture of hepatocytes, with cancerous foci and patchy necrosis. (F) Liver tissues from rats in the DEN-bearing CUR-treated group exhibited cancerous foci with focal necrosis. Stain, hematoxylin and eosin. Magnification, x400. DEN, N-nitrosodiethylamine; CUR, curcumin; GL63, (1E,4E)-1,5-bis(2-bromophenyl)penta-1,4-dien-3-one.

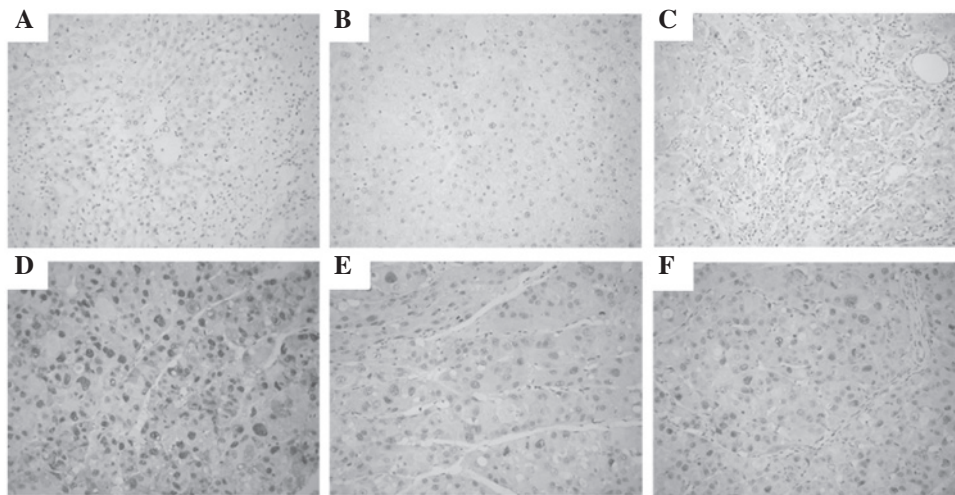


Figure 6. Immunohistochemical staining of PCNA in liver. (A) Liver from normal group displaying no PCNA staining pattern. (B) Hepatic tissues from rats treated with GL63 only exhibited very limited PCNA staining. (C) Hepatic tissues from rats treated with CUR only exhibited limited PCNA staining, similar to those of panel B. (D) Liver from rats in the DEN-positive control group displaying a number of colonies with strong PCNA staining. (E) Hepatic tissues from rats in the DEN-bearing GL63-treated group displaying moderate PCNA immunostaining. (F) The DEN-bearing CUR-treated group exhibited more PCNA staining than the DEN-bearing GL63-treated group. Magnification, x400. PCNA, proliferating cell nuclear antigen; DEN, N-nitrosodiethylamine; CUR, curcumin; GL63, (1E,4E)-1,5-bis(2-bromophenyl)penta-1,4-dien-3-one.

and varied (27). Several environmental carcinogens have been reported to elicit hepatic tumorigenesis (28).

Animal models such as Wistar rats can be used to simulate carcinogenesis and development of human hepatocarcinoma, and to study its molecular mechanism and intervention (29). DEN is a powerful carcinogen known to induce carcinoma in all animal species including humans, and is considered as an initiating agent in certain two-stage (initiation and promotion) protocols for hepatocarcinogenic studies (30). DEN-induced lesions and tumors in rodents exhibit marked biochemical, histological and molecular similarity to the progression of HCC in humans (31). DEN is an established powerful hepatocarcinogen in rats, possibly by altering the DNA structure, forming alkyl DNA adducts,

and inducing chromosomal aberrations and micronuclei in the liver (32,33).

Granado-Serrano *et al* reported a decrease in food intake, feed efficiency ratio and body weight gain, as well as a significant increase in relative liver weight, in the DEN group compared with the normal control group (34). The present results also confirmed this tendency. The reduction in food intake, and consequently, the reduction in body weight gain observed in DEN-treated animals may be due to losses of skeletal muscle and adipose tissue, with relative sparing of visceral proteins, as mentioned by Sreepriya and Bali (35), and it could be considered as an indirect indication of the declining hepatic function following exposure to DEN. Sivar-amakrishnan *et al* reported a marked loss in body weight and

an increase in liver weight induced by DEN in animals treated with DEN compared with the control group. In addition, the administration of DEN to animals also caused a significantly increase in liver weight due to the formation of nodules and tumors in the liver following carcinogen exposure (36). In accordance to the above report, the present study also observed a decrease in body weight and an increase in liver weight in animals treated with DEN. PCNA, as an index of the state of cell proliferation and indication of the malignant degree, was significantly enhanced in the nucleus of hepatocarcinoma cells, which has been confirmed in numerous studies (37). Therefore, enhanced expression of PCNA indicates abnormal proliferation of liver tissue. The present results demonstrated that GL63 treatment led to a decreased expression of PCNA in Wistar rats, which supported the preventive effects of GL63 against DEN-induced hepatocarcinogenesis in rats suggested by the present histopathological and immunohistochemical findings.

Due to its poor solubility and stability in water, the therapeutic benefit of CUR is limited (10). A series of mono-carbonyl analogues of CUR with higher potencies and improved water solubility, formulation and delivery system have been developed, since these properties are considered to be responsible for their stability *in vitro* and the pharmacokinetic advantages *in vivo* (20). In the present study, annexin V/PI staining demonstrated that, compared with CUR, GL63 increased the efficacy of induction of apoptosis in SK-HEP-1 cells. The results of flow cytometry also revealed that treatment with GL63 resulted in G0/G1 arrest of cancer cells, which may account for the inhibition of proliferation and cell cycle arrest exhibited by treated cells. Furthermore, the apoptotic rate induced by GL63 was higher than that induced by CUR. Based on these findings, a series of experiments were performed to further determine the potential apoptotic signaling pathways.

It is known that the dysregulation of the JAK/STAT signaling pathway may cause immunodeficiencies and cancers (38,39). Activated JAK2/STAT3 signaling pathway can modulate the expression of target genes that are involved in various physiological functions, including anti-apoptotic proteins (Bcl-x1 and Bcl-2), pro-apoptotic proteins (Bax and BH3 interacting-domain death agonist) and mitochondrial apoptosis pathway-related proteins (caspase-3, caspase-9, Cyt-c and PARP) (40). Importantly, the activated JAK2/STAT3 signaling pathway has been extensively validated as a novel molecular target for the treatment of human tumors (41). The present study further explored the role of the JAK2/STAT3 signaling pathway in the anti-hepatocarcinoma effect of GL63 for the first time, and evaluated that GL63 could efficiently inhibit the phosphorylation of JAK2 and STAT3, thus affecting cell viability and inducing apoptosis. GL63 can inhibit the JAK2/STAT3 signaling pathway with lower doses than those required for CUR, thus being a more efficient form of treatment for hepatocarcinoma than CUR. The present authors suggest that GL63 may have inhibitory effects on the proliferation of SK-HEP-1 cells, which may be mediated through the suppression of the JAK2/STAT3 signaling pathway.

Previous studies have demonstrated that the regulation of the JAK2/STAT3 signaling pathway by diverse drugs

can induce apoptosis through the intrinsic mitochondrial pathway (42). The intrinsic pathway is initiated at the level of the mitochondria under the regulation of the Bcl-2 family of proteins (43). Mitochondrial integrity and apoptosis are regulated mainly by the Bcl-2 family of cell-death regulatory molecules, which include the pro-apoptotic Bax and the anti-apoptotic Bcl-2 (44). Mitochondria mediate apoptotic signaling via the activation of the cell-death initiator pro-caspase-9 (45). Activated caspase-9 in turn cleaves executioner caspase-3 (46). The activated caspase-3 then cleaves PARP, a nuclear protein associated with the process of programmed cell death (47). The present data support the scenario that GL63 treatment induced apoptosis of SK-HEP-1 cells possibly through the caspase family proteins, leading to Cyt-c release and the activation of caspase-9, caspase-3 and PARP. It was observed that the antitumor activity of GL63 was also associated with the induction of apoptosis in HCC cells, as indicated by increased caspase-9, caspase-3 and PARP cleavage *in vitro*. In the present study, it was also revealed that the activation of caspase was involved in GL63-induced apoptosis, and a general caspase inhibitor markedly blocked GL63-induced cell death. These results suggested that GL63 induced apoptosis of SK-HEP-1 cells via a mitochondria-mediated intrinsic apoptosis pathway. Activation of the intrinsic pathway with GL63 was demonstrated by the activation of caspase-9 and caspase-3, along with the altered expression of Bcl-2 family proteins. In the current study, the significantly increased levels of Bcl-2 and Bcl-xL, and the activation of caspases-9 and -3, suggested that the intrinsic pathway is important in GL63-induced apoptosis in SK-HEP-1 cells. Compared with CUR, GL63 increased the efficacy of induction of apoptosis of SK-HEP-1 cells. GL63 treatment resulted in a reduction in JAK2 and STAT3 phosphorylation, activated caspase-9, caspase-3, Cyt-c and PARP cleavage, and caused apoptotic death of cells remarkably. This finding verifies previous studies reporting that inhibiting the JAK2/STAT3 signaling pathway is associated with the induction of apoptosis by the intrinsic mitochondrial pathway in hepatocarcinoma cells (48). It was also concluded that GL63 treatment inhibits hepatocarcinoma cell growth via downregulation of the JAK2/STAT3 signaling pathway.

The activity of GL63 was also evaluated in the present study using male Wistar rats, which is an animal model for studies of human hepatocarcinoma (49). For these *in vivo* studies, rats were divided into six different groups according to the requirements of the experimental design, and the animals were injected DEN into the peritoneum to induce hepatocarcinoma. A series of indexes of hepatocarcinoma were recorded for each group to judge the protective role of GL63 and CUR against hepatocarcinoma in rats. Although the results of the present study provide support for the chemopreventive effects of GL63 and CUR, GL63 could be a more potent compound than CUR for the prevention of DEN-induced hepatocarcinogenesis in rats.

In conclusion, the present results demonstrated that, compared with CUR, GL63 was more effective in inhibiting the JAK2/STAT3 signaling pathway and regulating the activation of the intrinsic mitochondrial pathway with a more favorable pharmacological activity than CUR, which contributed to the suppression of cell proliferation and the

induction of cell apoptosis. GL63 may have translational potential as an effective drug or preventive agent for hepatocarcinoma. The doses of GL63 that effectively demonstrate chemopreventive effects without toxicity for humans must be examined in future clinical studies.

Acknowledgements

The present study was supported by research grants from the Health and Family Planning Commission of Hebei Province (Shijiazhuang, China; grant no. 20150634) and the Tumor Research Institute, The Fourth Affiliated Hospital, Hebei Medical University (Shijiazhuang, China). The authors appreciate the generous support from Dr Yueping Liu, Dr Xiaoling Wang and Dr Huichai Yang for the pathological images.

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