

Digitoxin inhibits proliferation of multidrug-resistant HepG2 cells through G₂/M cell cycle arrest and apoptosis

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Abstract. Hepatocellular carcinoma (HCC) remains a challenge in the medical field due to its high malignancy and mortality rates particularly for HCC, which has developed multidrug resistance. Therefore, the identification of efficient chemotherapeutic drugs for multidrug resistant HCC has become an urgent issue. Natural products have always been of significance in drug discovery. In the present study, a cell-based method was used to screen a natural compound library, which consisted of 78 compounds, and the doxorubicin-resistant cancer cell line, HepG2/ADM, as screening tools. The findings of the present study led to the shortlisting of one of the compounds, digitoxin, which displayed an inhibitory effect on HepG2/ADM cells, with 50% inhibitory concentration values of 132.65±3.83, 52.29±6.26, and 9.13±3.67 nM for 24, 48, and 72 h, respectively. Immunofluorescence, western blotting and cell cycle analyses revealed that digitoxin induced G₂/M cell cycle arrest via the serine/threonine-protein kinase ATR (ATR)-serine/threonine-protein kinase Chk2 (CHK2)-M-phase inducer phosphatase 3 (CDC25C) signaling pathway in HepG2/ADM cells, which may have resulted from a DNA double-stranded break. Digitoxin also induced mitochondrial apoptosis, which was characterized by changes in the interaction between Bcl-2 and Bax, the release of cytochrome *c*, as well as the activation of the caspase-3 and -9. To

the best of our knowledge, the present study is the first report that digitoxin displays an anti-HCC effect on HepG2/ADM cells through G₂/M cell cycle arrest, which was mediated by the ATR-CHK2-CDC25C signaling pathway and mitochondrial apoptosis. Therefore, digitoxin could be a promising chemotherapeutic agent for the treatment of patients with HCC.

Introduction

Liver cancer, which is one of the most common tumors worldwide, has become difficult to treat due to its high malignancy and mortality rates (1). The mortality rate of liver cancer has increased from 7.5 to 11.2 in men and from 2.8 to 3.8 in women (per 100,000 persons) between 2000 and 2015, worldwide (2). A high amount of research has been performed to identify effective reagents for the treatment liver cancer (3). So far, chemotherapy remains the most widely used treatment for liver cancer; however, most conventional chemotherapeutic drugs, including cisplatin, 5-fluorouracil, and doxorubicin (Dox) exhibit poor efficiency in the treatment of hepatocellular carcinoma (HCC), with a <10% inhibition growth rate (4). A previous study has also confirmed the ineffectiveness of conventional chemotherapy whether they were administered intravenously or intra-arterially (5). Sorafenib[®] is the only tyrosine kinase inhibitor for HCC treatment approved by the Food and Drug Administration (FDA) of the United States of America; however, its limited efficacy and adverse side effects, including hand/food/skin reactions, asthenia, diarrhea, and arterial hypertension, highlights whether it is suitable for use in a clinical setting (5,6).

Multidrug resistance (MDR) also contributes to the intractability of HCC. MDR is defined as a process in which cancer cells gain resistance to multiple chemotherapeutic drugs with different structures and mechanisms of action (7). MDR has been reported to be responsible for >90% of cases in which chemotherapy had failed and the tumor had recurred (8). Therefore, identification of efficient drugs to combat MDR has become an important issue in the medical field. Natural products have great potential for drug discovery and constitute

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a large number of chemotherapy agents in cancer treatment. For example, the discovery of vinblastine and vincristine have been developed for use as anticancer agents from natural sources (9). An increasing number of compounds derived from natural resources have been approved as anticancer drugs by the FDA, such as camptothecin, paclitaxel, anthracyclines and taxanes (10-12). Among these, drugs which induce cancer cell cycle arrest or apoptosis are a big part. It is widely accepted that cell cycle arrest may result from DNA damage (13). ATM, a serine/threonine protein kinase, activates checkpoint signaling upon DNA double-stranded breaks (DSBs), thereby acting as a DNA damage sensor and playing a significant role in cell cycle arrest (14).

Digitoxin is a natural cardiac glycoside derived from *Digitalis* (15). As a potent inhibitor of Na⁺/K⁺-ATPase, digitoxin has been clinically used for congestive heart failure for more than 40 years (16). Previously, a number of studies have focused on the anticancer potential of digitoxin and verified notable antitumor activities of digitoxin in lung cancer (17), pancreatic cancer (18), glioma (19), liver cancer (20), prostate cancer (21) and melanoma (22). Mechanistic studies have revealed that the growth inhibitory effect of digitoxin was associated with the induction of apoptosis (23), inhibition of epithelial-mesenchymal transition (21) and suppression of cancer cell stemness (24); however, the underlying mechanism of action of digitoxin against multidrug-resistant HCC cells has not been fully elucidated.

In the present study, a library of 78 natural compounds, including digitoxin was screened in the Dox-resistant cancer cell line, HepG2/ADM. Further investigations demonstrated that digitoxin displayed an inhibitory effect on multidrug-resistant HepG2/ADM cells through G₂/M cell cycle arrest via the serine/threonine-protein kinase ATR (ATR)-serine/threonine-protein kinase Chk2 (CHK2)-M-phase inducer phosphatase 3 (CDC25C) signaling pathway and mitochondrial apoptosis. The findings of the present study suggested that digitoxin may be developed into a chemotherapeutic agent for patients with HCC.

Materials and methods

Reagents and antibodies. A library of 78 natural compounds was obtained from Target Molecule Corp. Digitoxin (≥98% pure) was purchased from Baoji Herbest Bio-Tech Co., Ltd. MTT was supplied by Sigma-Aldrich (Merck KGaA). An Annexin-V-FITC/propidium iodide (PI) staining assay kit was obtained from Beyotime Institute of Biotechnology. The bicinchoninic protein assay kit (BCA) was purchased from Thermo Fisher Scientific Inc., while PI and 4',6-dimidyl-2-phenylindole (DAPI) were purchased from Roche Diagnostics (Shanghai) Co. Ltd. Primary antibodies against cyclin-dependent kinase 1 (CDK1, #9116), cyclin B1 (#4138), phosphorylated (p)-CDK1 (Thr14) (#2543), p-histone H2AX (γH2AX, #9718), ATR (#2790), p-ATR (Ser428) (#2853), CHK2 (#6334), p-Chk2 (Thr68) (#2197), CDC25C (#4688), p-CDC25C (Thr48) (#12028), Bax (#5023), Bcl-2 (#15071), cytochrome *c* (#11940), caspase-9 (#9508) and -3 (#9662), cleaved-caspase-3 (#9579) and -9 (#20750), cleaved poly (ADP-ribose) polymerase (PARP) (#5625), β-actin (#4970) and the horse-radish peroxidase (HRP)-conjugated secondary antibodies (Anti-mouse

IgG, #7076; Anti-rabbit IgG, #7074), Alexa Fluor 647-conjugated anti-rabbit IgG (H+L) (#4414) were obtained from Cell Signaling Technology Inc., (dilution of primary antibodies, 1:1,000; dilution of secondary antibodies, 1:2,000).

Cell line and cell culture. The Dox-resistant human HCC cell line, HepG2/ADM was provided by Professor Kwok-Pui Fung (The Chinese University of Hong Kong, Hong Kong, China). HepG2/ADM cells were cultured in RPMI 1640 medium supplemented with Dox (1.2 μM, Sigma-Aldrich), 1% penicillin-streptomycin (PS), and 10% fetal bovine serum (FBS) to maintain the multidrug-resistant characteristics of the HepG2/ADM cell line. RPMI 1640 medium, PS, and FBS were supplied by Thermo Fisher Scientific Inc.. Cells were incubated at 37°C in a humidified incubator with 5% CO₂.

Compound library screening. The cytotoxicity screening of the 78 natural compounds in the library against HepG2/ADM cells was performed via the MTT assay. Cells (5,000/well) were seeded into 96-well plates and cultured overnight at 37°C. After treatment with 78 natural compounds (0.1 μM) for 72 h at 37°C, respectively, cells were incubated with 20 μl MTT (5 mg/ml) at 37°C for 3 h. The formazan crystals were dissolved in 100 μl dimethylsulfoxide (DMSO) and the absorbance of each well was recorded at 595 nm wavelengths using a microplate reader (Beckman Coulter Inc.).

Cell viability assay. Viability of HepG2/ADM cells was determined using a MTT assay. Cells (5,000/well) were seeded in 96-well plates and cultured overnight. Following treatment with digitoxin at concentrations ranging from 3.906-1,000,000 nM for 24, 48 and 72 h, respectively, cells were exposed to 20 μl MTT (5 mg/ml) and incubated at 37°C for 3 h. The formazan crystals were dissolved with 100 μl DMSO and the absorbance was measured at 595 nm using a microplate reader (Beckman Coulter Inc.). As previously described (25), cells treated with medium containing 0.2% DMSO for 24, 48 or 72 h were considered as 100% viable, respectively.

Cell cycle analysis. HepG2/ADM cells (3x10⁵/well) were seeded in 6-well plates and cultured overnight, then treated with digitoxin at 4, 20 and 100 nM for 24 h or 20 nM digitoxin for 12, 24 and 36 h, respectively. Following fixation and permeabilization with pre-cooled 75% ethanol at 4°C overnight, cells were stained with 0.2 mg/ml PI and 0.1 mg/ml RNase in the dark at room temperature for 15 min. The PI fluorescence of the cells was analyzed using an EPICS-X flow cytometry (Beckman Coulter, Inc.). Then, the phase distribution of cell cycle was analyzed using ModFit LT v3.1 software (Verity Software House, Inc.).

Western blot analysis. Following treatment with digitoxin for 24 h, HepG2/ADM cells were collected using trypsinization. Total cellular protein was extracted using the radioimmunoprecipitation assay (RIPA) lysis buffer (containing 1 mM PMSF, 1X phosphatase inhibitor and 1X protease inhibitor, Beyotime Institute of Biotechnology). Then, a BCA assay kit was used to quantify the protein concentration. Proteins (30 μg/lane) were separated using 12% SDS-PAGE gels and then transferred onto PVDF membranes. The membranes were blocked with

5% skimmed milk at room temperature for 1 h and probed with the primary antibodies at a dilution of 1:1,000 overnight at 4°C. After incubation with secondary antibody at a dilution of 1:2,000 for 1 h at room temperature, the protein bands were visualized using an ECL detection kit (Millipore, Merck KGaA) and quantified using the ImageJ software v1.8.0 (National Institutes of Health). β -actin was used as the loading control.

Immunofluorescence. HepG2/ADM cells were treated with digitoxin for 24 h. Then, cells were fixed with 4% paraformaldehyde (PFA) for 15 min at room temperature. After blocking with 5% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA) containing 0.4% Triton X-100 (Sigma-Aldrich; Merck KGaA) for 1 h at room temperature, the cells were incubated with the γ H2AX primary antibody (1:1,000) overnight at 4°C and Alexa Fluor 647-conjugated secondary antibody (1:2,000) for 1 h at room temperature. Fluorescence was observed using a confocal microscope with a 40x magnification (Axio Vert.A1; Zeiss GmbH).

Annexin-V-FITC/PI staining assay. Following treatment with digitoxin for 24 and 48 h, respectively, HepG2/ADM cells were collected and stained with Annexin-V-FITC/PI for 15 min in darkness at room temperature. The cell apoptotic rates were analyzed using an Epics XL flow cytometer (Beckman Coulter Inc.). The following wavelengths were used 488 (excitation) and 525 (emission) nm for Annexin V-FITC; and 488 (excitation) and 620 nm (emission) for PI. The data was quantified using the FlowJo v7.6 software (FlowJo LLC).

Statistical analysis. All experiments were performed at least three times. Results are presented as the mean \pm SEM. GraphPad Prism v7.0 (GraphPad Software Inc.) was used for statistical analysis. One-way analysis of variance (ANOVA) followed by a Tukey's post hoc test was used for multiple comparison. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Digitoxin shows cytotoxicity towards HepG2/ADM cells. The cytotoxicity of 78 natural compounds on HepG2/ADM cells were determined using the MTT assay. As shown in Fig. 1A, digitoxin (No. 63; chemical structure shown in Fig. 1B) was found to have a greater cytotoxic effect on HepG2/ADM cells. Subsequently, the anti-HCC effect of digitoxin on HepG2/ADM cells was assessed using the MTT assay. As shown in Fig. 1C, digitoxin decreased the viability of HepG2/ADM cells in a dose-dependent manner, with IC_{50} values of 132.65 ± 33.83 , 52.29 ± 6.26 and 9.13 ± 3.67 nM following treatment for 24, 48 and 72 h, respectively. The concentrations used in the cell viability assay were based on the results of the pre-experiment.

Digitoxin blocks the HepG2/ADM cell cycle at G₂/M phase. To investigate whether the inhibitory effect of digitoxin on HepG2/ADM cells was associated with cell cycle arrest, DNA content analysis was performed using flow cytometry. The cell population at G₂/M phase increased from 23.61 to 46.87% with 0, 12, 24, and 36 h (0 h as the CTL group) following

20 nM digitoxin treatment, while 0, 4, 20, and 100 nM digitoxin treatment (0 nM as the CTL group) for 24 h also resulted in an increase in the number of cells at G₂/M phase from 23.64 to 41.40%, indicating that digitoxin induced G₂/M cell cycle arrest in HepG2/ADM cells (Fig. 2A and B). CDK1 and cyclin B1, two key regulators of G₂/M transition, have been found to be involved in modulating the cell cycle by forming the CDK1/Cyclin B1 complex (26). Digitoxin treatment caused significant downregulation of p-CDK1 (Thr14) and accumulation of cyclin B1 compared with the CTL group, which further confirms that digitoxin blocked the HepG2/ADM cell cycle at G₂/M phase (Fig. 2C and D).

It is well-known that cell cycle arrest may result from DNA damage, in which the serine-protein kinase (ATM)/ATR-CHK1/CHK2-CDC25C signaling pathway plays significant role (27). To determine whether DNA lesions were responsible for the G₂/M cell cycle arrest induced by digitoxin, an immunofluorescent staining assay was performed to detect the expression level of γ H2AX, a marker of DNA double-stranded break (DSB) (28). The numbers of punctuate γ H2AX foci significantly increased in a dose-dependent manner following digitoxin treatment compared with the CTL group. The highest accumulation of γ H2AX foci was detected following treatment with 100 nM digitoxin (Fig. 3A and B). ATM and ATR are pivotal sensors of the DNA damage response pathway (29) and regulate the cell cycle partly through activating cell cycle checkpoint kinases CHK1 and CHK2 (30). Active CHK1 and CHK2 decrease the activity of CDC25C, thus inhibiting the dephosphorylation of CDK1 to maintain the inactive status of the CDK1-cyclin B1 complex (31,32). Digitoxin significantly increased the protein expression levels of p-CHK2 (Thr68) in a dose-dependent manner as well as p-ATR (Ser428) compared with the CTL group. In addition, digitoxin inhibited the phosphorylation of CDC25C (Fig. 3C and D). Taken together, these results indicated that digitoxin induced G₂/M phase arrest via the ATR-Chk2-Cdc25C signaling pathway following DNA damage.

Digitoxin induces HepG2/ADM cell apoptosis through the mitochondrial apoptotic pathway. To investigate the underlying mechanism of digitoxin-induced HepG2/ADM cell death, the Annexin V-FITC/PI staining flow cytometry assay was performed to determine whether digitoxin induced apoptosis in HepG2/ADM cells. Different concentrations (CTL, 4, 20, 100 and 500 nM) of digitoxin treatment resulted in a significant augmentation of apoptotic cells (Fig. 4A and B). The apoptotic ratio of HepG2/ADM cells increased by approximately 7-fold from 5.68 to 43.15% for 24 h and 13-fold from 5.71 to 73.74% for 48 h. In addition, the ratio of cleaved caspase-3/caspase-3 and cleaved caspase-9/caspase-9 increased following different concentrations of digitoxin in a dose-dependent manner, and the level of cleaved PARP also increased, confirming that the apoptosis-related genes in the caspase family were activated (Fig. 4E and F). Mitochondrial apoptosis is regarded as the most common mode of cell apoptosis, which is characterized by the release of cytochrome *c* and changes in the interaction between Bcl-2 (apoptosis inhibitor) and Bax (apoptosis promotor) (33,34). As expected, digitoxin slightly increased the protein expression level of Bax and notably

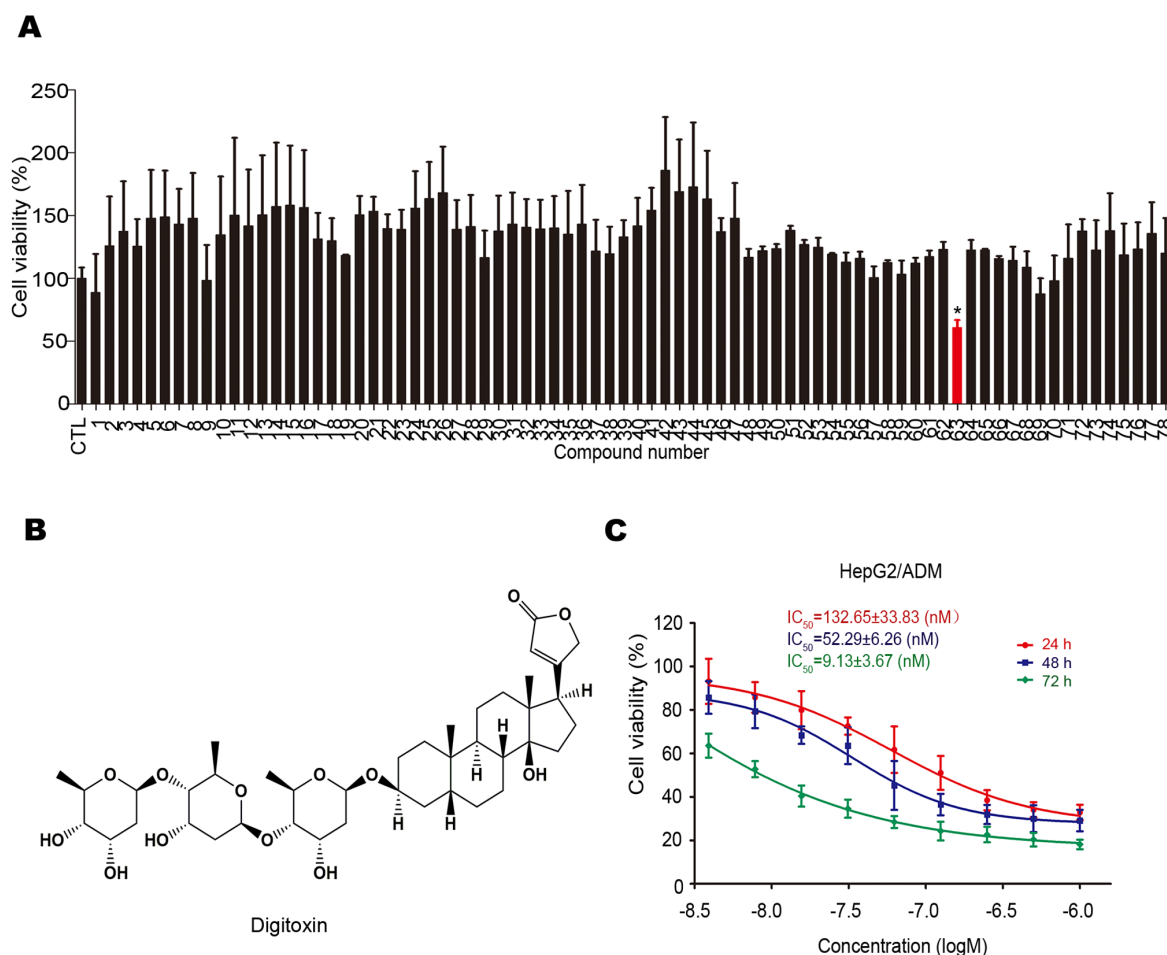


Figure 1. Digitoxin shows cytotoxicity towards HepG2/ADM cells. (A) HepG2/ADM cells were treated with 78 compounds at 0.1 μ M for 24 h and the viability was measured using the MTT assay. Digitoxin (No. 63) was identified as the most cytotoxic compound towards HepG2/ADM cells among the different compounds, indicated by the red color. * $P < 0.05$ vs. the control group. (B) Chemical structure of digitoxin. (C) HepG2/ADM cells were treated with digitoxin at different concentrations for 24, 48 or 72 h and the viability was measured using the MTT assay. Digitoxin exerted an anti-HCC effect on HepG2/ADM cells in a dose- and time-dependent manner.

decreased the expression level of Bcl-2. As a consequence, the value of Bax/Bcl-2 increased approximately 58-fold by 20 nM digitoxin treatment compared with the CTL group. Since the level of Bcl-2 after 20 nM digitoxin treatment is very low, higher concentrations of digitoxin were not used in this assay. In addition, digitoxin treatment increased the protein expression level of cytochrome *c* (Fig. 4C and D). Taken together, these results indicated that digitoxin induced HepG2/ADM cell apoptosis through the mitochondrial apoptotic pathway.

Discussion

Resistance to chemotherapy is the primary problem for effective treatment in patients with liver cancer (35). A high amount of research has been performed to overcome the problem of multidrug resistance (36-38); however, no important breakthroughs have been achieved (39). Thus, there remains an urgent requirement to identify novel anticancer agents that are effective against chemotherapy-resistant tumors. In the present study, a Dox-resistant HCC cell line, HepG2/ADM was used to screen a library of 78 natural compounds, in which digitoxin was selected due to its effective anti-HCC action on HepG2/ADM cells. In addition, the molecular and/or cellular mechanisms

of the apoptotic effect of digitoxin on HepG2/ADM cells were also investigated. The findings of the present study demonstrated that digitoxin induced G₂/M cell cycle arrest via the ATR-CHK2-CDC25C signaling pathway, which may result from DNA DSB. Furthermore, digitoxin was found to induce mitochondrial apoptosis in HepG2/ADM cells. The notable findings of the present study indicate that digitoxin could be a potential novel anti-HCC drug, particularly in chemotherapy-resistant HCC.

Digitoxin, a cardenolide, is an inhibitor of Na⁺/K⁺-ATPase (16). A number of studies have revealed the anticancer activities of digitoxin against various human cancer cell lines, including hematological, solid, drug-sensitive and/or drug-resistant cancer cells *in vitro* and *in vivo* (21,40-42). In liver cancer, a previous study found that the combination of sorafenib and digitoxin significantly inhibited primary HepG2 cell growth, which was potentially through suppression of ERK and hypoxia signaling (43). To the best of our knowledge, no study has addressed the effect of digitoxin on Dox-resistant HepG2/ADM cells. The present study performed purely *in vitro* work. *In vivo* studies are required to investigate digitoxin efficacy in detail, and whether ERK and hypoxia signaling pathways play a role in digitoxin-induced HepG2/ADM cell death also requires further investigation.

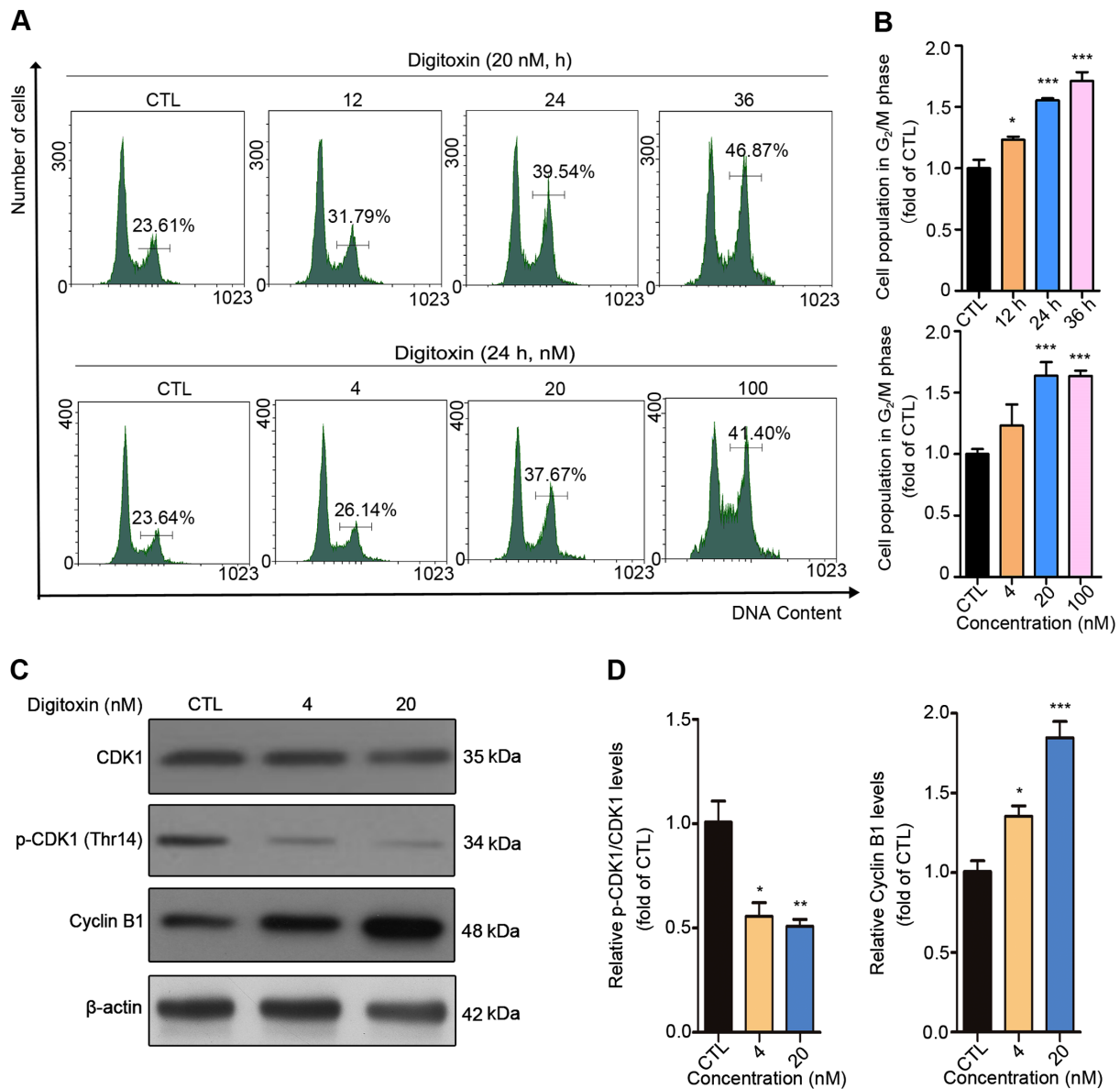


Figure 2. Digitoxin blocks HepG2/ADM cells in the G₂/M phase of the cell cycle. (A) HepG2/ADM cells were treated with different concentrations of digitoxin (0, 4, 20, 100 nM) for 24 h or 20 nM of digitoxin for 0, 12, 24 and 36 h, then the cell cycle distributions were detected using flow cytometry. The cell population in the G₂/M phase was increased following digitoxin treatment. (B) The cell population in the G₂/M phase was quantified using Prism. Each column represents the mean ± SEM (n=3). *P<0.05, ***P<0.001 vs. the control group. (C) HepG2/ADM cells were treated with or without digitoxin (4 and 20 nM) for 24 h, and the protein expression levels of CDK1, p-CDK1 (Thr14) and cyclin B1 were measured using western blot analysis. β-actin served as the loading control. Digitoxin-induced G₂/M phase arrest was associated with CDK1 and cyclin B1. (D) Quantitative analysis of the relative protein expression. Data are presented as the mean ± SEM. *P<0.05, **P<0.01, ***P<0.001 vs. the control group. CTL, control; CDK1, cyclin-dependent kinase 1; p, phosphorylated.

Numerous studies performed by different laboratories have demonstrated that digitoxin induces G₂/M cell cycle arrest in several human cancer cell lines, including the KG1a acute myelogenous leukemia cell line and the K562 chronic myelogenous leukemia cell line (41), 786-O and A498 renal cell carcinoma cell lines (44), and the non-small cell lung cancer cell lines, NCI-H460 (45) and H1975 (17). Previous mechanistic studies have revealed that the downregulation of the cyclin B1/CDK1 complex, and the protein expression levels of CHK1/2 and p53, with the decrease in the protein levels of E3 ubiquitin-protein ligase Ccnb1ip1, cyclin-A1, p21, p27, c-Myc and p-5AMP-activated protein kinase catalytic subunit α-2 have been associated with digitoxin-induced G₂/M cell cycle arrest (17,45). The findings of the present

study demonstrated cell cycle arrest at the G₂/M phase in HepG2/ADM cells following digitoxin treatment; however, reduced level of p-CDK1 (Thr14) and accumulation of cyclin B1 caused by digitoxin were detected in the present study (Fig. 2C and D), which may promote cell cycle progression according to the majority of studies (27,46). In eukaryotic cells, the expression level of cyclin B1 is very low in the G₁ phase, and significantly increases during the S phase and peaks at the late G₂ phase and early mitosis. When cells enter late mitosis, the expression level of cyclin B1 was found to be significantly decreased (47-51). Therefore, in the present study the increase of cyclin B1 further confirms that a higher proportion of HepG2/ADM cells were in the G₂/M phase following digitoxin treatment. With respect to decreasing level

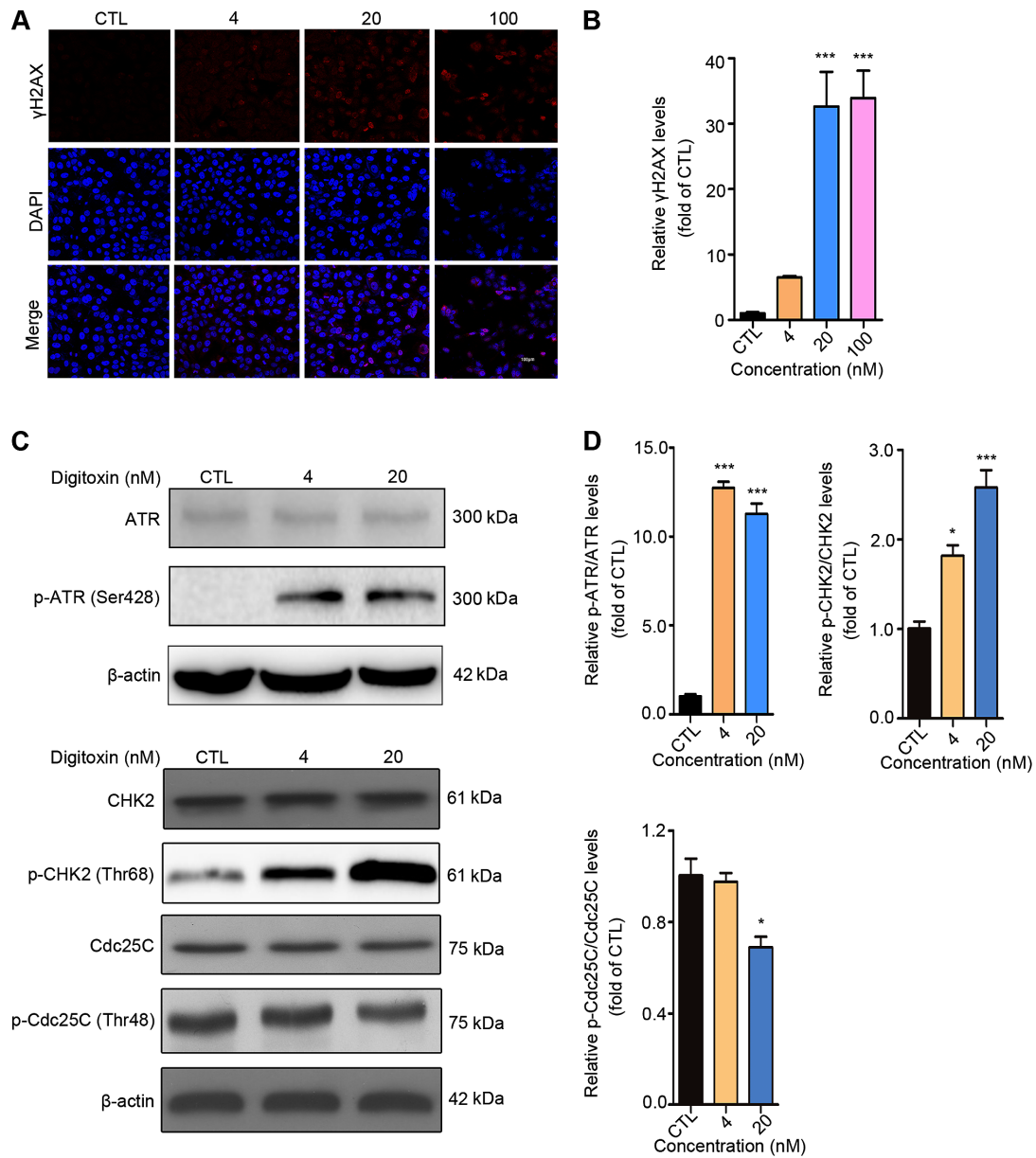


Figure 3. Digitoxin induces G_2/M phase arrest via the ATR-CHK2-CDC25C signaling pathway. (A) Digitoxin increased the expression level of γ H2AX. HepG2/ADM cells were treated with or without digitoxin (4, 20 and 100 nM) for 24 h, then, the expression level of γ H2AX was measured using an immunofluorescence assay. Images were obtained at a magnification of $\times 200$. (B) γ H2AX fluorescence intensity was subsequently quantified. Data are shown as the mean \pm SEM ($n=3$). $^{***}P<0.001$ vs. the control group. (C) The effect of digitoxin on the ATR-CHK2-CDC25C signaling pathway. HepG2/ADM cells were treated with or without digitoxin (4 and 20 nM) for 24 h, then western blot analysis was used to detect the protein expression levels of ATR, p-ATR (Ser428), CHK2, p-CHK2 (Thr68), CDC25C, p-CDC25C (Thr48). β -actin was used as the loading control. (D) The relative protein expression was quantified. Data are presented as the mean \pm SEM ($n=3$). $^*P<0.05$, $^{***}P<0.001$ vs. the control group. γ H2AX, p-histone H2AX; ATR, serine/threonine-protein kinase ATR; CHK2, serine/threonine-protein kinase chk2; CDC25C, M-phase inducer phosphatase 3; p, phosphorylated; CTL, control.

of p-CDK1 (Thr14), some signaling pathways, which are activated to regulate cell cycle events such as P21 and P53 (52,53) may be responsible for this phenomenon. Similar results have been reported in other studies. Lee *et al* (54) demonstrated that p-CDK1 dephosphorylation at Tyr15 and the upregulation of cyclin B1 expression level were detected in 2-methoxyestradiol-induced G_2/M arrest in Jurkat T cells. Mak *et al* (55) reported that small-molecule inhibitors of CHK1 (AZD7762) or WEE1 (MK-1775) induced mitotic arrest, as characterized by the dephosphorylation of p-CDK1 (Tyr15) in HeLa cells. Rong *et al* (56) also found that p-CDK1 was dephosphorylated at Thr161 following gambogic acid-induced DNA damage and

G_2/M arrest in HepG2 and A549 cells. However, the reasons for these different effects of digitoxin are complex and require further investigation.

A number of small molecules can arrest the cell cycle at G_1/S or S phase to prevent incorrect DNA replication or at G_2/M phase to prevent entry into mitosis with damaged DNA (57). The present study found that digitoxin impeded cell cycle progression at the G_2/M phase, suggesting that digitoxin may not block DNA replication but induce DNA damage instead. In addition, the activation of the DNA damage response ATR-CHK2-CDC25C pathway and the increase of γ H2AX (Ser139) were also found, which confirmed that the molecular

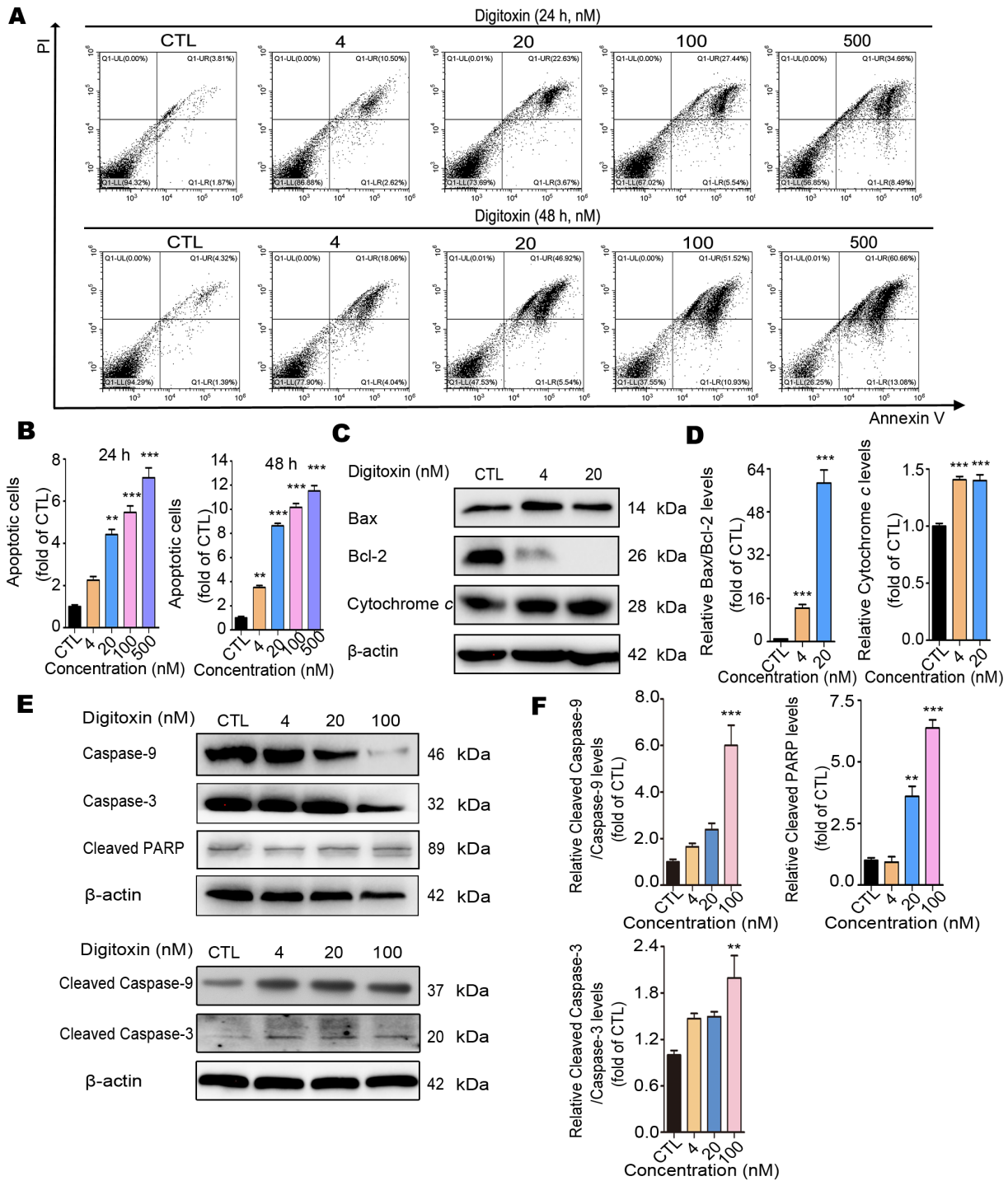


Figure 4. Digitoxin induces HepG2/ADM cell apoptosis via the mitochondrial apoptotic pathway. (A) Following treatment with different concentrations (0, 4, 20, 100 and 500 nM) of digitoxin for 24 or 48 h, the apoptotic ratio of HepG2/ADM cells was determined using the Annexin V-FITC/PI staining assay. Digitoxin induced HepG2/ADM cell apoptosis. (B) The data of apoptotic cells was quantified and illustrated as the mean \pm SEM (n=3). **P<0.01, ***P<0.001 vs. the control group. (C) The effect of digitoxin on the protein expression levels of mitochondrial apoptosis-related proteins. Following treatment with or without digitoxin (4 and 20 nM) for 24 h, the expression levels of Bax, Bcl-2 and cytochrome c were determined using western blot analysis. β -Actin was used as the loading control. (D) The relative protein expression levels were quantified, and the data are presented as the mean \pm SEM (n=3). ***P<0.001 vs. the control group. (E) The effect of digitoxin on the expression levels of the apoptosis-related proteins. Following treatment with or without digitoxin (4, 20 and 100 nM) for 24 h, the expression levels of caspase-3 and -9, cleaved-caspase-3, and -9, and cleaved PARP were detected using western blot analysis. β -actin was used as the loading control. (F) The relative protein expression levels were quantified, and the data are presented as the mean \pm SEM (n=3). **P<0.01, ***P<0.001 vs. the control group. PARP, cleaved poly (ADP-ribose) polymerase; PI, propidium iodide; CTL, control group.

mechanism of modulating the cell cycle by digitoxin was induction of DNA damage.

Previous studies have demonstrated that Na⁺/K⁺-ATPase was considered as a potential target for cardenolides to

combat some cancers (58,59). The protein expression levels of Na⁺/K⁺-ATPase in tumor tissues, such as HCC, renal carcinoma cells, non-small cell lung carcinoma, colon carcinoma, prostate carcinoma, and glioma was higher compared with

that in normal tissues (60,61). In addition, the colocalization of Na⁺/K⁺-ATPase and caveolin on the plasma membrane induced by the knockdown of apolipoprotein E increased the sensitivity of Hep3B cells towards cardenolides, confirming the role of Na⁺/K⁺-ATPase in the cytotoxicity of cardenolides (20). These findings demonstrate that the anticancer effects of cardenolides were associated with Na⁺/K⁺-ATPase. However, to the best of our knowledge there is a shortage of literature demonstrating that digitoxin-induced cell cycle arrest was attributed to the inhibition of Na⁺/K⁺-ATPase. Therefore, the exact molecular mechanism of how digitoxin induces DNA DSB, as well as whether Na⁺/K⁺-ATPase inhibition was responsible for apoptotic effect of digitoxin in HepG2/ADM cells requires further investigation. Furthermore, *in vivo* effects and cardiotoxicity of digitoxin also require further investigation.

In conclusion, our study demonstrated that digitoxin displays an anti-HCC effect on HepG2/ADM cells through ATR-CHK2-CDC25C-mediated G₂/M cell cycle arrest and Bax/Bcl-2-mediated mitochondrial apoptosis, making digitoxin a promising chemotherapeutic agent for the treatment of patients with HCC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

LJD and FFX designed the study and revised the manuscript for important intellectual content. YHL and HG performed the experiments and drafted the manuscript. YQH, JYZ, HZ, MSW, XJL and QYM made contributions to analysis and interpretation of data. LC and AYS performed the flow cytometry experiments and analyze the data. JW, YXL, EXZ and YYC assisted with the revision of the manuscript and performed experiments to update the data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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