Upregulation of cyclin-dependent kinase inhibitors CDKN1B and CDKN1C in hepatocellular carcinoma-derived cells via goniothalamin-mediated protein stabilization and epigenetic modifications

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ABSTRACT

Cell cycle deregulation is common in human hepatocellular carcinoma (HCC). To ensure proper cell cycle controlling, cyclin/cyclin-dependent kinases (CDK) complexes are tightly regulated by CDK inhibitors (CKIs) in normal cells. However, insufficient cyclin-dependent kinase inhibitor 1B (CDKN1B, also known as p27^{Kip1}) and CDKN1C (p57^{Kip2}) proteins are characteristics of high-risk HCC. In two HCC-derived cell lines with distinct genetic backgrounds, we identified a small natural compound, goniothalamin (GTN), serving as an inducer of CKIs. In TP53-mutated (Y220C) and retinoblastoma 1 (RB1)-positive Huh-7 cells, GTN stabilized CDKN1B protein levels by targeting the degradation of its specific E3 ubiquitin ligase (S-phase kinase-associated protein 2). Alternatively, in TP53- and RB1-negative Hep-3B cells, GTN increased CDKN1C transcription and its subsequent translation by acting as a histone deacetylase inhibitor. In both cell lines, GTN induced G_0/G_1 cell cycle arrest, delayed S phase entry of cells and inhibited anchorage-independent cell growth which might be attributed to the upregulation of CKIs and downregulation of several positive cell cycle regulators, including CDC28 protein kinase regulator subunit 1B, cyclin E1 and D1, cyclin-dependent kinase 2 (CDK2), CDK4, CDK6, E2F transcription factor 1 and/or transcription factor Dp-1. Therefore, GTN might represent a novel class of anticancer drug that induces CKIs through post-translational and epigenetic modifications.

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1. Introduction

Hepatocellular carcinoma (HCC) is the most common primary malignant neoplasm of the liver worldwide, accounting approximately 6% of all human cancers annually (reviewed in [39]). Although substantial advances have been made in chemotherapy regimens for HCC, the efficacy of drugs is often hampered by a range of adverse side effects (reviewed in [53]). Clinical and genetic heterogeneity observed in HCC patients further complicates the use of general therapies (reviewed in [19]). Accordingly, there remains a critical need for both continued molecular characterization and aggressive drug development in HCC (reviewed in [16]).

Cell cycle deregulation is common in human HCC. Many studies have highlighted that G_1 phase-associated cell cycle regulators might be closely implicated in the early steps of carcinogenesis (reviewed in [10]). Two major cyclin/cyclin-dependent kinase (CDK) complexes are involved in the progression of G_1: cyclin D/CDK4 or CDK6 complex for early G_1 phase and cyclin E/CDK2 complex for late G_1 to S phase. These cyclin/CDK complexes are required for hyperphosphorylation of the retinoblastoma 1 (RB1), leading to the transcription of genes implicated in S phase progression. Conversely, G_1 phase-related cyclin-CDK complexes are negatively regulated by a series of CDK inhibitors (CKIs) in normal cells. Diminished expression of cyclin-dependent kinase inhibitor 1B (CDKN1B, also known as p27) [20,32] and cyclin-dependent kinase inhibitor 1C (CDKN1C, p57) [15,37] predict poor prognosis in the hepatocellular carcinoma. It is generally believed that CDKN1B is degraded via the ubiquitin-proteasome system (UPS) in cancer cells [34,41], while CDKN1C is inactivated via either UPS or epigenetic modifications [6].

Studies performed over the past two decades by us and other researches have shown that, goniothalamin (GTN), a plant bioactive styryllactones mainly isolated from the genus *Goniothalamus*, is cytotoxic to a variety of tumor cell lines including those from the breast, blood, ovary, cervical, lung, kidney, prostate, hepatoblast, colon and liver [1–3,9,11,22,23,26,28,45]. Besides, GTN is more cytotoxic to cancer than to normal cells [52].

Numerous studies also showed that GTN induced apoptosis via caspase-dependent (CASP2, -3, -7, -8 and/or -9) pathways in different carcinoma-derived cells including cervix (HeLa) [4], promyelocytic leukemia (HL-60), histiocytic lymphoma (U937) [21], leukemia (Jurkat T) [22,23], hepatocellular carcinoma (Hep-G2) [1] as well as coronary artery smooth muscle cells [8]. Undeniably, DNA damage response of mammalian cells usually involves cell cycle arrest and DNA repair or, if unsuccessful, cell death (reviewed in [40]). We earlier identified that GTN induces the formation of reactive oxygen species (ROS)/DNA double-strand breaks (DNA damage), transactivation of tumor protein p53 (TP53) and/or pophorbol-12-myristate-13-acetate-induced protein 1 (*PMAIP1*) gene, translocation of TP53 and/or PMAIP1 protein to mitochondria, release of cytochrome *c* from mitochondria, cleavages of caspase 8 (CASP8), -9, -3, poly (ADP-ribose) polymerase 1, and induction of apoptosis, sequentially, in both TP53-positive and -negative HCC cell lines [26]. However, prior to the occurrence of apoptosis, how GTN dysregulated cell cycle progression remained unclear. We herein identified that GTN induced G_0/G_1 cell-cycle arrest by upregulation of two CKIs, CDKN1B and CDKN1C, in two distinct HCC-derived cell lines, the underlying regulatory mechanisms were also studied.

2. Materials and methods

2.1. Cell culture

Two HCC-derived cell lines, Huh-7 and Hep-3B, were maintained in a humidified incubator with 5% CO_2 atmosphere at 37 °C in Dulbecco's Modified Eagle's Medium and Medium Essential Medium (CORNING), respectively, supplemented with 10% fetal bovine serum, 1% L-glutamine (2 mM), 1% nonessential amino acids, 1 mM sodium pyruvate, 50 IU/mL penicillin and 50 μg/mL streptomycin (Sigma–Aldrich). These two cell lines were known to have distinct genetic backgrounds. The Huh-7 is characterized by expression of mutated TP53 (Y220C), cyclin-dependent kinase inhibitor 1A (CDKN1A, i.e., p21[Cip1]), RB1 proteins, and the absence of hepatitis B virus surface antigen, while pertinent features of these markers are complete opposite in Hep-3B cells, including the absent of *TP53* gene [11,44].

2.2. Chemicals

GTN was prepared as our previous study [27] dissolved in dimethyl sulfoxide (DMSO). The maximum amount of DMSO in culture medium was 1/1000. All chemicals unless otherwise stated were purchased from Sigma–Aldrich. N-(1H-Benzotriazol-1-yl)-2,4-dichlorobenzamide (ITSA1, CAS 200626-61-5), an histone deacetylase (HDAC) activator by suppression of trichostatin A (TSA) [25] was obtained from Santa Cruz Biotechnology. Cycloheximide (CHX), MG132 (a proteasome inhibitor), 5-Aza-2′-deoxycytidine (5-Aza, an epigenetic modifier inhibits DNA methyltransferase), TSA and ITSA1 were dissolved in DMSO. All working solutions were freshly prepared from stocks.

2.3. Flow cytometry

Literally 5×10^5 cells were treated with DMSO (control) or GTN (Huh-7: 20 μM; Hep-3B: 15 μM) for 24 h, collected, washed with ice-cold PBS twice, fixed with 80% ethanol and stored at −20 °C. Before analysis, fixed cells were washed with ice-cold PBS for three times, treatments with 200 μg/mL RNase A (#78020Y, Affymetrix) and 20 μg/mL propidium iodide for 3 h. To analyze the cell cycle distribution, a total of 10,000 events were examined using a Coulter® Epics® XL™ Flow Cytometer (Beckman Coulter) and the Modfit software (BD Biosciences).

2.4. Soft agar assay

CytoSelect™ 96-Well In Vitro Tumor Sensitivity Assay (soft agar colony formation, CBA-150, Cell

Biolabs, Inc.) was used to analyze GTN-mediated alterations in colony formation, followed by the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the manufacturer's instructions to evaluate the cell viability. Briefly, 50 µL/well (in a 96-well sterile flat-bottom microplate) of the Base Agar Matrix Layer was prepared by mixing 1.25 mL of 2× DMEM/20% FBS medium, 1 mL of sterile water, 0.25 mL of melted 10× CytoSelect^{TM} Agar Matrix Solution. Cell Suspension/Agar Matrix Layer under sterile conditions (75 µL/well) was made by mixing 1.75 mL of 2× DMEM/20% FBS medium, 1.375 mL of CytoSelect^{TM} Matrix Diluent, 0.375 mL of melted 10× CytoSelect^{TM} Agar Matrix Solution and 0.25 mL of Cell Suspension. Literally 10^3 and 10^5 cells of Huh-7 and Hep-3B were added in the upper agar and incubated for 8 and 9 days, respectively.

2.5. Immunoblotting analysis

Cell lysates were prepared with RadioImmunoPrecipitation Assay Buffer (#9806, Cell Signaling). Lysates containing equal amounts of protein were separated by 8, 10, 12 or 15% SDS-polyacrylamide gel electrophoresis as appropriate and electroblotted onto the polyvinylidene fluoride membrane (Immobilon^{TM} Transfer Membrane; Millipore). The filters were individually probed with the primary anti-human antibodies, including anti-human β actin (ACTB; 1:3000, #MAB1501, Millipore), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 1:1000, #ab128915, Abcam), cyclin-dependent kinase 2 associated protein 1 (CDK2AP1, i.e., p12; 1:500 [54]), cyclin-dependent kinase inhibitor 2B (CDKN2B, p15^{Ink4b}; 1:500, sc-612, Santa Cruz), CDKN2C (p18^{Ink4c}; 1:500, sc-865, Santa Cruz), CDKN2D (p19^{Ink4d}; 1:500, sc-1063, Santa Cruz), CDKN1A (p21^{Cip1}; 1:1000, #2947, Cell Signaling), CDKN1B (p27^{Kip1}; 1:500, #3686, Cell Signaling), CDKN1C (p57^{Kip2}; 1:500, sc-1040, Santa Cruz), CDC28 protein kinase regulatory subunit 1B (CKS1B; 1:200, #36-6800, Invitrogen), cyclin E1 (CCNE1; 1:500, sc-198, Santa Cruz), cyclin-dependent kinase 2 (CDK2; 1:500, sc-163, Santa Cruz), CCND1 (1:1000, #2926, Cell Signaling), CDK4 (1:500, sc-260, Santa Cruz), CDK6 (1:500, sc-177, Santa Cruz), RB1 (1:500, sc-102, Santa Cruz), E2F transcription factor 1 (E2F1; 1:500, sc-251, Santa Cruz), transcription factor Dp-1 (TFDP1; 1:500, sc-53642, Santa Cruz), S-phase kinase associated protein 2, E3 ubiquitin protein ligase (SKP2; 1:500, #32-3300, Invitrogen), poly(ADP-ribose) polymerase 1 (PARP1; 1:1000, #9542, Cell Signaling), APC/C$^{Fizzy/cell}$ $^{division\ cycle\ 20\ related\ 1\ (FZR1)}$ (FZR1 E3 ligase, also known as CDH1; 1:1000, GTX-111200, Genetex), acetyl-histone H3 (1:10,000, #06-599, Merck Millipore), acetyl-histone H4 (#06-866, Merck Millipore) and histone H3 containing the acetylated lysine 9 and 14 (H3K9/14ac; 1:10,000, GTX-122648, Genetex) antibodies. Protein bands were detected by Western Lightning^{TM} Chemiluminescence reagent Plus ECL Kit (PerkinElmer), with horseradish-peroxidase-labeled secondary antibodies as suggested by the manufacturer and visualized on a VersaDoc^{TM} Imaging System (Bio-Rad Life Science). The intensity of bands from immunoblotting assays was quantified by densitometry and normalized to that of ACTB or GAPDH in each lane.

2.6. Quantitative reverse transcription-polymerase chain reaction

Total RNAs were extracted with TRIzol$^®$ isolation reagent (Life Technologies) from cells followed by DNase treatment and reverse transcription to synthesize cDNA with Moloney Murine Leukemia Virus Reverse Transcriptase (Promega). TaqMan$^®$ chemistry with primers and probes (Life Technologies): CDKN1B (#4331182; 151 bp, Hs01597588_m1, NM_004064.3), CDKN1C (#4331182; 91 bp, Hs00175938_m1, NM_000076.2, NM_001122630.1, NM_001122631.1), SKP2 (#4331182; 59 bp, Hs01021864_m1, NM_001243120.1, NM_005983.3, NM_032637.3) and GAPDH (#4453320; 93 bp, Hs02758991_g1, NM_001256799.1, NM_002046.4) were used for quantitative RT-PCR. The relative expression folds of each target transcript were given by $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_T$ (treatment) − ΔC_T (DMSO); ΔC_T represented the C_T of a target transcript subtracted from the C_T of GAPDH (internal control). Only samples with C_T value < 25 for GAPDH were considered to meet acceptable RNA quality standards and included in the analyses.

2.7. CHX-chase assay

A standard CHX-chase assay was used to evaluate whether CHX-inhibited CDKN1B protein synthesis could be restored by GTN treatments. Briefly, Huh-7 cells (3×10^5) were grown in 6-well plates and treated with DMSO (control) and GTN (10 µM) for 24 h, respectively. Afterward, CHX (25 µg/mL) was added and cells were harvested at 0, 2, 4, 8 h and subjected to immunoblotting analysis.

2.8. Nuclear/cytosol fractionation

To examine changes of nuclear SKP2, CDK2 and CDKN1B protein levels after GTN treatments, nuclear/cytosol fractionation along with immunoblotting were performed. A total of 10^6 Huh-7 cells were seeded in 10-cm tissue culture dishes and treated with DMSO (control) or GTN for 16 and 24 h, respectively. Nuclear/Cytosol Fractionation Kit (K266-25, BioVision Inc.) was used to isolate nucleus and cytosol using trypsinized cells according to the manufacturer's instructions.

2.9. Histone extraction

The EpiSeeker Histone Kit (#ab113475, Abcam) was used to isolate the histone protein. Briefly, 10^6 of Hep-3B cells were plated in 10-cm culture dishes. After TSA or GTN treatments, trypsinized cells were resuspended with 1×, 100 µL Pre-lysis Buffer in microtubes and incubated on ice for 10 min. Cells were centrifuged at $9000 \times g$, 4 °C for 1 min, and the supernatant was removed. The cells were next resuspended in 20 µL of Lysis Buffer and incubated on ice for 30 min. Cells were centrifuged again at

13,000 × g, 4 °C for 5 min and the supernatant was transferred into new microtubes. Literally 60 μL of Balance-DTT Buffer was added to balance the pH value and protein concentrations were determined for immunoblotting analysis.

2.10. Statistics

Student's t-test was used to examine the significant difference between the averages of the control and the experimental group.

3. Results

3.1. GTN induced G_0/G_1 cell cycle arrest and inhibited anchorage-independent cell growth in HCC-derived cells via upregulation of CDKN1B and CDKN1C expression

Half maximal inhibitory concentrations (IC_{50}) of GTN in Huh-7 and Hep-3B cells were determined as 20 and 15 μM, respectively (at 48 h) by the MTT assay. To analyze GTN-induced cell cycle arrest at the earliest, these concentrations were used for subsequent experiments. Flow cytometric analysis showed that GTN treatments for 24 h induced G_0/G_1 cell cycle arrest ($P < 0.01$) and decreased cell numbers in the S phase ($P < 0.001$) in both Huh-7 and Hep-3B cell lines (Fig. 1A and E). Soft agar assays further demonstrated that GTN treatments suppressed colony formation (Fig. 1B and F) and anchorage-independent cell growth ($P < 0.001$) in both the cell lines (Fig. 1C and G). Accordingly, the protein levels of a series of CKIs, which control G_1 to S progression including CDK2AP1, CDKN2B, CDKN2C, CDKN2D, CDKN1A, CDKN1B and CDKN1C were investigated. Immunoblotting showed GTN-mediated upregulation of CDKN1B and CDKN1C protein levels in Huh-7 and Hep-3B cells, respectively, among the seven CKIs examined (Fig. 1D and H). Therefore, GTN induces G_0/G_1 cell cycle arrest inhibits anchorage-independent cell growth in HCC-derived cells via upregulation of CDKN1B and CDKN1C protein levels. How GTN induced CDKN1B and CDKN1C were further studied.

3.2. GTN upregulated CDKN1B and CDKN1C but downregulated regulators of G_1 to S phase in distinct HCC-derived cells

Immunoblotting analysis and a time-course experiment identified the optimal GTN induction periods as 16 h ($P < 0.05$) and 24 h ($P < 0.05$) for CDKN1B and CDKN1C protein levels in Huh-7 and Hep-3B cells, respectively (Fig. 2A and C). Several G_1- and S-phase regulatory proteins, upstream or downstream of CKIs, including CKS1B, CCNE1, CCND1, CDK2, CDK4, CDK6, RB1, E2F1, and/or TFDP1 were downregulated after GTN treatments for 24 h (Fig. 2B and D). Accordingly, GTN upregulates CDKN1B and CDKN1C protein levels, accompanying by downregulation of several regulators involving in the transition between G_1 to S phase of the mitotic cell cycle in distinct HCC-derived cell lines.

3.3. GTN stabilized nuclear CDKN1B protein via degradation of its E3 ligase, SKP2 protein level in Huh-7 cells

In Huh-7 cells, GTN treatments for 24 h notably upregulated CDKN1B but downregulated its E3 ligase, SKP2 protein level (Fig. 3A). In contrast, CDKN1B and SKP2 mRNA levels were not altered after GTN treatments in time-course experiments ($P > 0.05$; Fig. 3B), signifying that GTN increased SKP2 protein stability. Moreover, GTN augmented and stabilized CHX-suppressed SKP2 and CDKN1B protein levels, respectively, implying that GTN might be an SKP2 inhibitor (Fig. 3A). A proteasome inhibitor, MG132, stabilized the endogenous SKP2 and reversed the GTN-inhibited SKP2 protein expression (Fig. 3C), suggesting that SKP2 levels might also be regulated by the UPS pathway in this cellular context. However, while GTN suppressed SKP2 protein level, it did not regulate the coactivator of the anaphase-promoting complex, FZR1, which targets SKP2 for degradation in the G_1 phase (Fig. 3D). Together with immunoblotting analysis, cytosol/nuclear fractionation demonstrated that nuclear CDKN1B protein level was upregulated ($P < 0.05$) whereas SKP2 ($P < 0.01$) and CDK2 ($P < 0.01$) levels were downregulated following GTN treatments, indicating that GTN induces nuclear CDKN1B expression by suppression of SKP2, and subsequently, downregulates CDK2 protein levels (Fig. 3E). Thus, GTN induces the CDKN1B protein level in Huh-7 cells by targeting CDKN1B-specific E3 ligase, SKP2, for degradation.

3.4. GTN-induced CDKN1C mRNA levels via epigenetic modifications in Hep-3B cells

In Hep-3B cells, a time-course experiment showed that GTN treatments did not markedly alter SKP2 protein level ($P > 0.05$; Fig. 4A) but upregulated CDKN1C mRNA level ($P < 0.01$; Fig. 4B), indicating that GTN enhances CDKN1C transcription. As shown in Fig. 4C, TSA rather than 5-Aza-dC treatments, upregulated CDKN1C mRNA level ($P < 0.001$), further demonstrating that inhibition of HDAC upregulates CDKN1C mRNA level in Hep-3B cells. In addition, compared to treatment with TSA or GTN alone, combined treatment with GTN and TSA upregulated CDKN1C mRNA levels ($P < 0.001$). On the other hand, cotreatment with TSA, GTN, and 5-Aza-dC did not further upregulate CDKN1C mRNA level as compared to cotreatment with TSA and GTN ($P > 0.05$). However, the mRNA levels of CDKN1C induced by the cotreatment of GTN and TSA were not immediately reflected in their protein levels (Fig. 4D).

To further compare the roles between GTN- and TSA-induced CDKN1C mRNA, a TSA-specific inhibitor, ITSA1 was used. Treatment with ITSA1 alone suppressed endogenous CDKN1C mRNA level ($P < 0.01$), indicating that the low CDKN1C mRNA level in Hep-3B cells was inhibited by the endogenous HDAC. Interestingly, GTN restored the ITSA1-suppressed CDKN1C mRNA to the original level (Fig. 4E); this effect was however delayed at the protein level (Fig. 4F). Furthermore, immunoblotting analysis showed that GTN upregulated global acetyl-histone H3 but not acetyl-H4; this finding was similar to that observed in the TSA-treated group (Fig. 4G). A slight but distinct
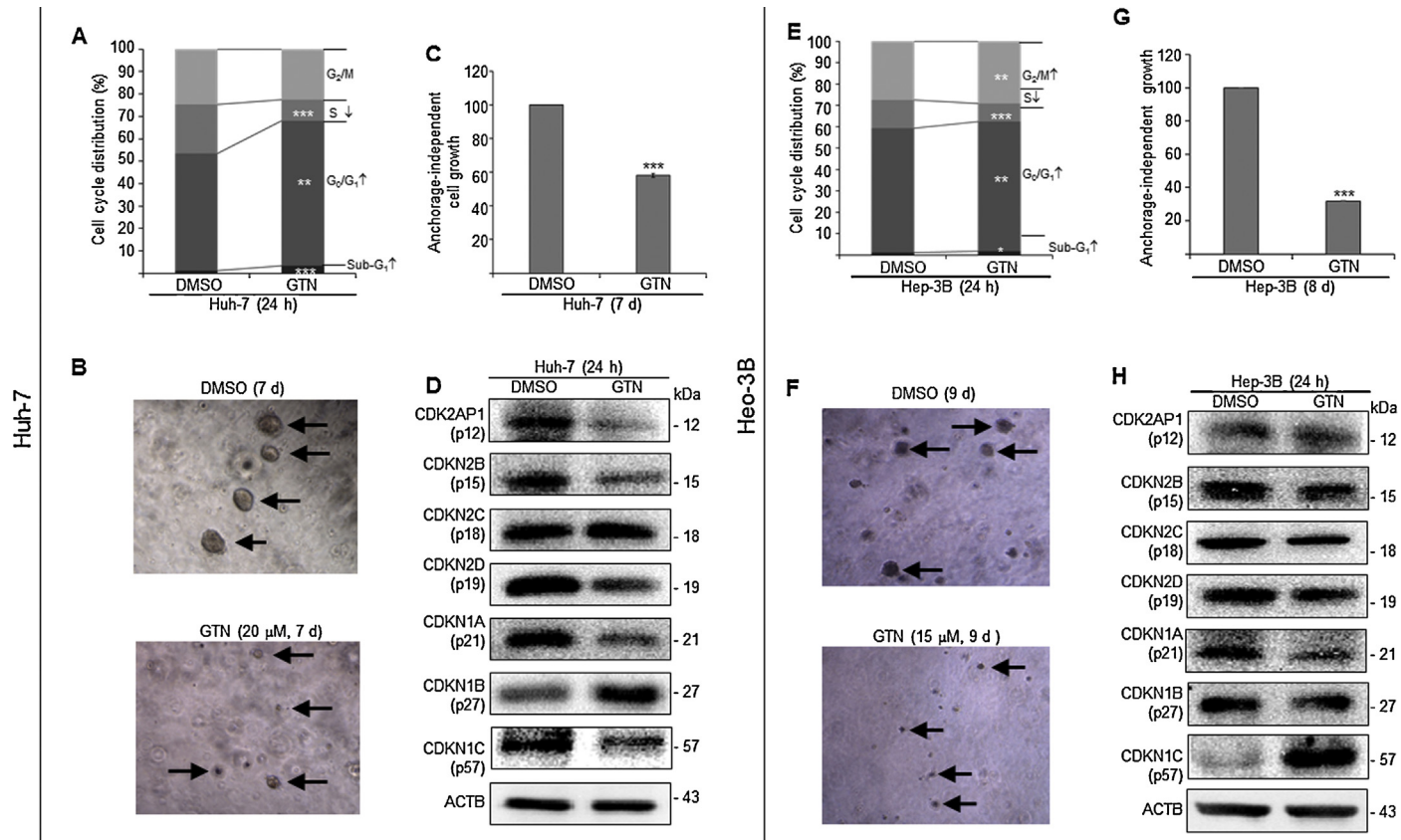
Fig. 1. Goniothalamin (GTN) induced cell cycle arrest and suppressed anchorage-independent cell growth via upregulation of cyclin-dependent kinase inhibitor 1B (CDKN1B) and CDKN1C protein levels in HCC-derived cells. Flow cytometric assays demonstrated that treatment with GTN in TP53-mutated Huh-7 (20 μM) and TP53-negative Hep-3B (15 μM) cells for 24 h induced G_0/G_1 and/or G_2/M cell cycle arrest; suppressed S-phase and increased sub-G_1 apoptotic cells. Soft agar assays were performed for 7 d and 9 d in TP53-mutated Huh-7 and TP53-negative Hep-3B cells, respectively. Compared to DMSO control GTN-induced the inhibition of anchorage-independent cell growth (B, F). MTT assay further identified viable cells were decreased after GTN treatments in both cell lines (C, G). Screening on a series of cyclin-dependent kinase inhibitors (CKIs) by immunoblotting analyses identified that CDKN1B and CDKN1C were notably upregulated in Huh-7 and Hep-3B cells, respectively, after treatments with GTN for 24 h, while the expression levels of other CKIs were downregulated or unchanged. All experiments were triplicated and representative immunoblotting images are shown. Statistical significance: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.
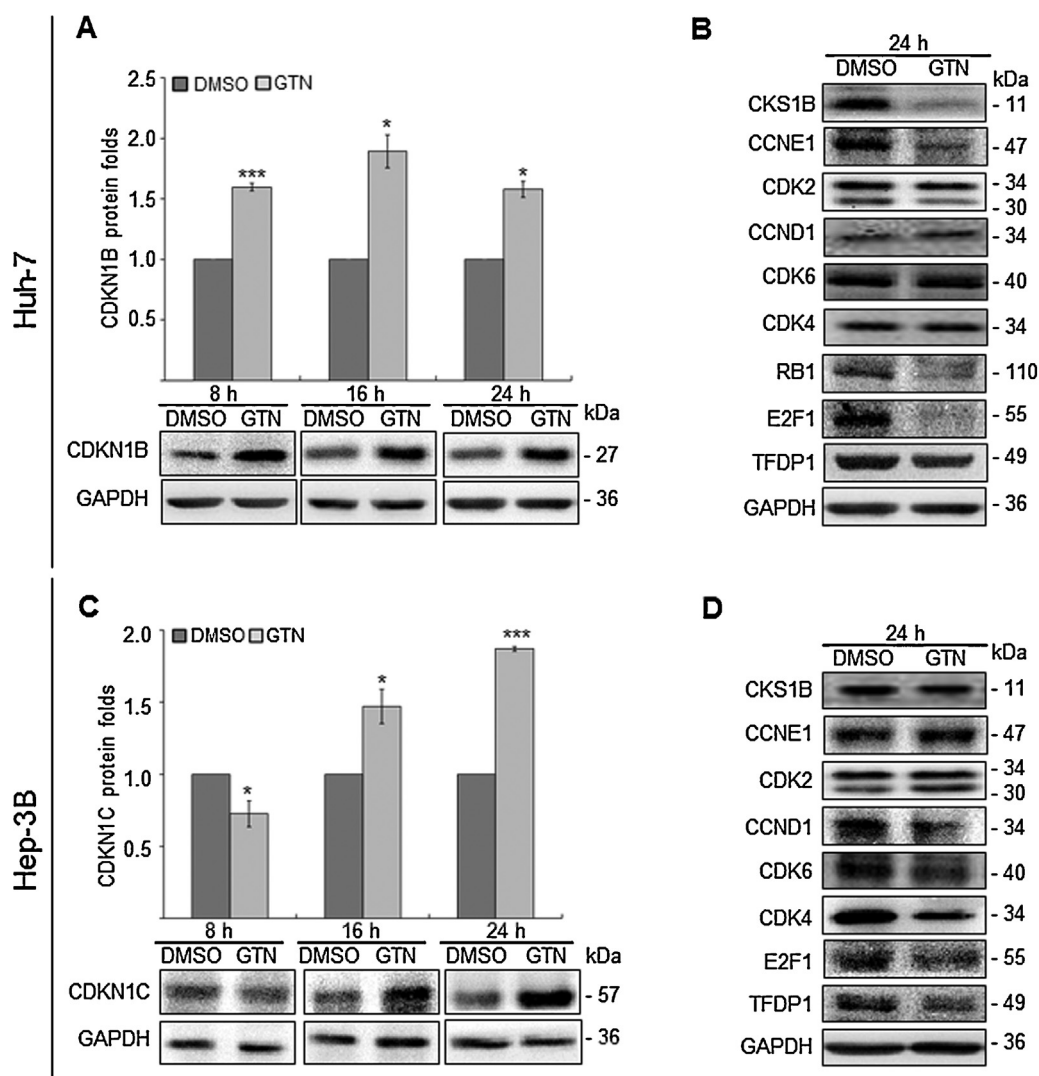
Fig. 2. While Goniothalamin (GTN) increased the protein abundance of CDKN1B and CDKN1C, positive regulator proteins of G_1 to S phase were downregulated in Huh-7 and Hep-3B cells. Immunoblotting analysis demonstrated that (A, C) CDKN1B and CDKN1C protein levels were significantly upregulated after GTN treatments for 8, 16 and 24 h in Huh-7 cells; for 16 and 24 h in Hep-3B cells, respectively. (B, D) Of several CDKN1B and CKDN1C upstream and downstream G_1 and S regulators, GTN notably suppressed CKS1B, CCNE1, CDK2, RB1, E2F1 and TFDP1 protein levels in Huh-7; CDK2, CCND1, CDK6, CDK4, E2F1 and TFDP1 protein abundance in Hep-3B cells.

upregulation of H3K9/14 acetylated protein was found after TSA or GTN treatment (Fig. 4H). Consequently, GTN induces *CDKN1C* mRNA level by playing a similar role to that of TSA in Hep-3B cells. The underlying molecular mechanisms of GTN-upregulated CDKN1B and CDKN1C in distinct HCC-derived cells are summarized in Fig. 5.

4. Discussion

This study demonstrated that a small natural compound, GTN, induced G_0/G_1 cell cycle arrest and inhibited anchorage-independent cell growth by modulating two potent CKIs, CDKN1B and CDKN1C, in HCC-derived cell lines with distinct genetic backgrounds. Uncontrolled proliferation is one of the main hallmarks of human cancers

[46]. In many cancers, including HCC [33], hyperactivity of CDKs is one of the mechanisms underlying the physiological hyperproliferation. Therefore, inhibition of CDKs has emerged as a potential therapy and upregulation of endogenous CKIs has become an alternative strategy to inhibit hyperproliferation. This study indicates GTN as a potent CKI inducer.

We found that after treatments with GTN for 24 h in Huh-7 (20 μM) and Hep-3B (15 μM) cells, only a few were identified in sub-G_1 (i.e., apoptosis) while most (60–70%) cells were arrested in G_0/G_1 phase. Therefore, GTN inhibits the G_1/S transition point via upregulation of the protein levels of two CKIs, namely CDKN1B and CDKN1C. Members of the Cip/Kip family of CKIs are well characterized for their role as negative regulators of G_1-phase cell cycle
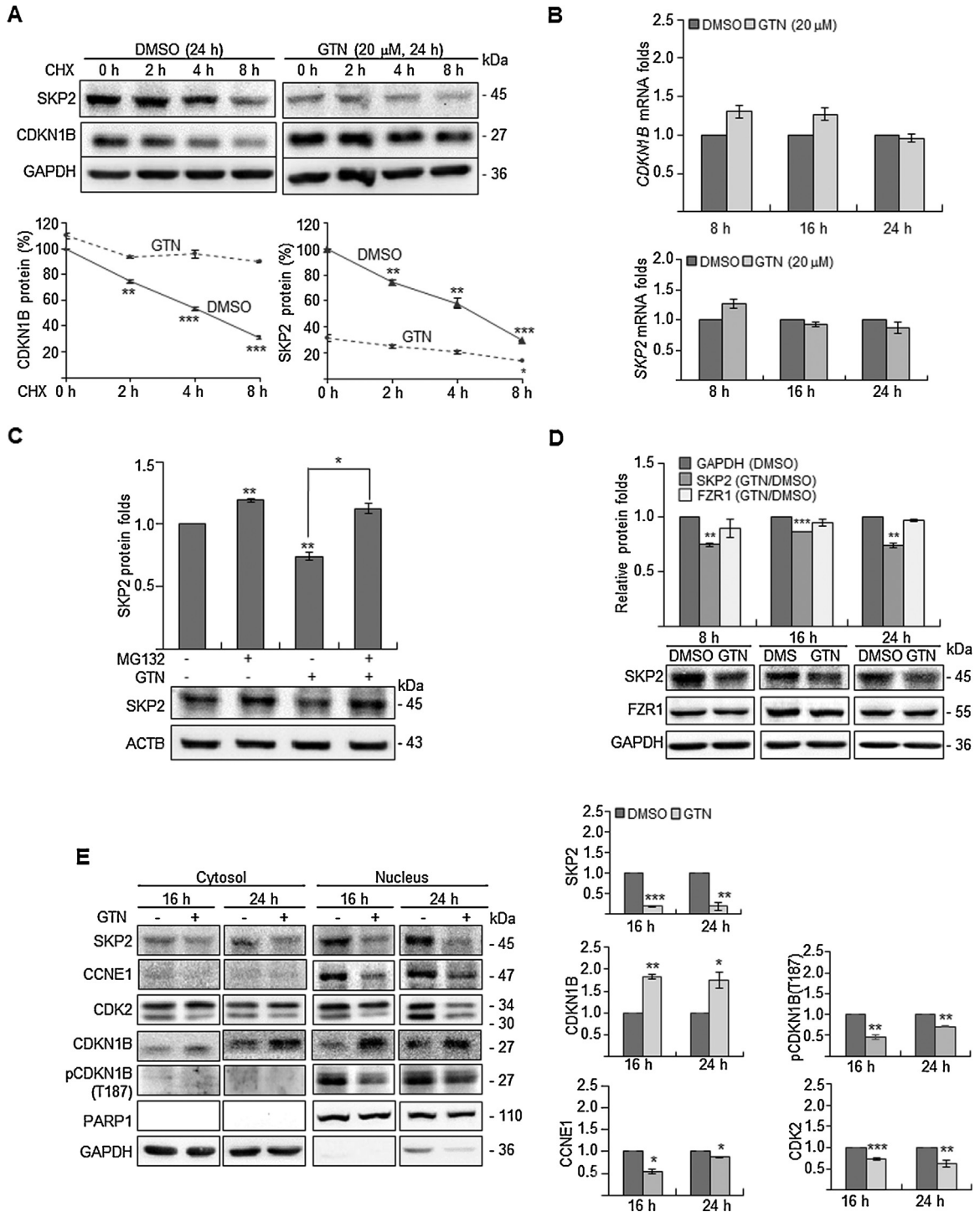
Fig. 3. In Huh-7 cells, goniothalamin stabilized nuclear CDKN1B via downregulation of its E3 ligase SKP2 level. (A) GTN treatment for 24 h upregulated CDKN1B while downregulated SKP2 protein level [DMSO 24 h/Cycloheximide (CHX) 0 h vs. GTN 24 h/CHX, 0 h]. CHX-chase (0, 2, 4, 8 h) along with immunoblotting assays estimated that the half-life of CDKN1B and SKP2 proteins were approximately 6 h. Compared to the DMSO group, GTN treatments prolonged the stabilities of high CDKN1B and low SKP2 protein levels. (B) GTN treatments did not alter *CDKN1B* and *SKP2* mRNA levels in a time course experiment, compared to their corresponding controls (DMSO). (C) MG132 (5 μM) increased SKP2 protein abundance and further restored GTN-inhibited SKP2 protein level. DMSO (control), GTN and MG132 were added at time 0, 0 and 16 h, respectively; the total reaction time was 20 h. (D) GTN treatments for 8, 16 and 24 h downregulated SKP2, nevertheless, did not alter FZR1 (an E3 ligase of SKP2) protein abundance. (E) Fractionation of nuclear and cytosolic proteins and immunoblotting analysis indicated that GTN notably induced nuclear CDKN1B, however, downregulated nuclear SKP2, CCNE1, CDK2, pCDKN1B(T187) protein levels. PARP1 and GAPDH were served as nuclear and cytosolic control, respectively. All experiments were triplicated and results are expressed as mean ± SEM. Representative immunoblotting images are shown. Statistical significance: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
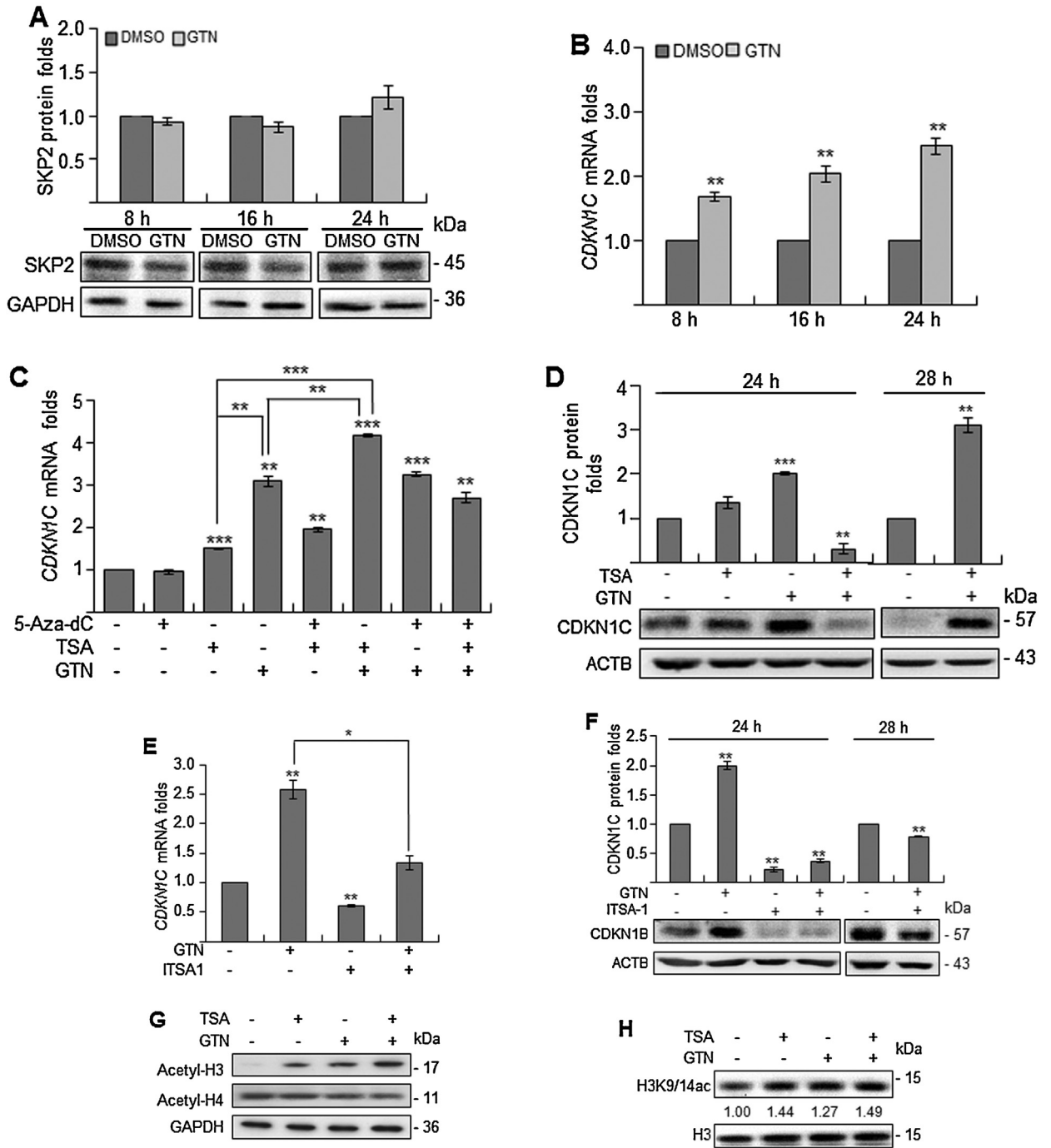
Fig. 4. In Hep-3B cells, goniothalamin induced *CDKN1C* mRNA and subsequent protein levels via upregulation of acetyl-H3 and H3K9/14ac levels. Immunoblotting identified that SKP2 (an CDKN1C-specific E3 ligase) protein level was almost unchanged after GTN treatments for 8, 16 and 24 h. (B) Quantitative RT-PCR showed that GTN upregulated *CDKN1C* mRNA levels in a time-dependent manner. (C) Treatments with trichostatin A (TSA; 100 ng/mL) and GTN (15 μM) for 24 h but not 5-Aza-2′-deoxycytidine (5-Aza-dC; 10 μM) for 72 h, upregulated *CDKN1C* mRNA level, compared to the control group (DMSO). Combined treatments of TSA and GTN further upregulated *CDKN1C* mRNA level, compared to either treatment with TSA or GTN alone. (D) CDKN1C translation was delayed to 28 h when combined treatments with TSA and GTN, compared to its transcription level at 24 h. (E, F) ITSA1 (50 μM, 24 h), a TSA-specific inhibitor, downregulated endogenous and GTN-induced *CDKN1C* mRNA and protein levels, compared to the controls (DMSO). Similarly, CDKN1C translation was delayed to 28 h when combined treatments with GTN and ITSA1. ITSA1 further downregulated GTN-induced *CDKN1C* mRNA and protein levels. (G) Immunoblotting analysis demonstrated that TSA and GTN notably upregulated acetyl-H3 but not acetyl-H4 protein level, with an additive effect. (H) Histone extraction and immunoblotting analysis further demonstrated that both TSA and GTN increased H3K9/14-acetylated proteins in Hep-3B cells, with a slightly additive effect. All experiments were triplicated and results are expressed as mean ± SEM. For immunoblotting analysis, representative images are shown. GAPDH, ACTB and H3 were served as loading controls. Statistical significance: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.
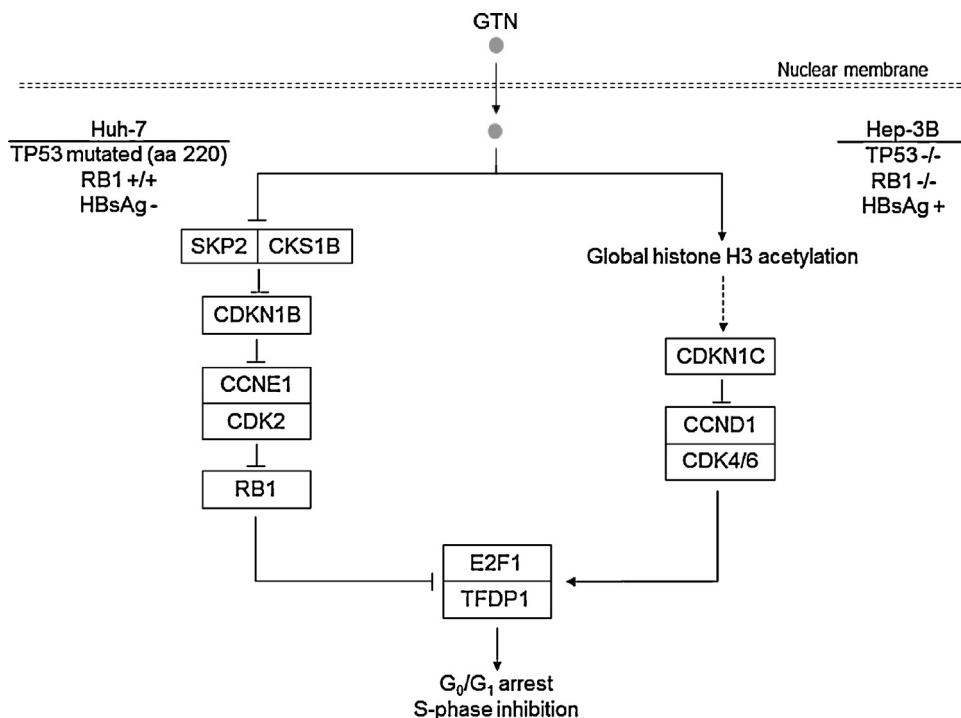
Fig. 5. Summary on the goniothalamin (GTN)-induced cyclin-dependent kinase inhibitors (CKIs) in distinct HCC-derived cell lines.

progression. Based on the sequence homology and specificity of action, CKIs are divided into two distinct families: INK4 and Cip/Kip [48]. Members of the INK4 family, namely CDKN2B, CDKN2A, CDKN2C and, CDKN2D, specifically inhibit the activity of CDK4 and CDK6, whereas Cip/Kip members, i.e., CDKN1A, CDKN1B, and CDKN1C inhibit a broader spectrum of cyclin-CDK complexes [14,17,29,43,50]. Therefore, GTN notably induces Cip/Kip members of CKIs targeting spacious cyclin-CDK complexes rather than the INK4 family in distinct HCC-derived cells.

Among all the CKIs examined, CDKN1B and CDKN1C protein levels were particularly low in Huh-7 and Hep-3B cells, respectively; GTN-induced CDKN1B and CDKN1C expressions strongly correlated with decrement of the rate of cell cycle entry at G_1/S transition, anchorage-independent cell growth and colony formation. The cell cycle is regulated primarily by the activity of CDKs and protein degradation by the UPS. In the S phase, ubiquitination of nuclear CDKN1B is triggered by phosphorylation of threonine 187 [pCDKN1B(T187)] through CDK2-cyclin E/A complexes when the number of CDK2-CCNE/A complexes exceeds that of the available CDKN1 [18,36,47]. Then, nuclear pCDKN1B(T187) is recognized by the ubiquitin-ligase SKP1-cullin 1 (CUL1)-ring-box1, E3 ubiquitin protein ligase (RBX1)-F box protein (SCF) SKP2 E3 ligase, i.e., SCF^{SKP2}, and targets pCDKN1B(T187) for proteasomal degradation [5]. In this process, CKS1B has been identified as an essential cofactor [49]. SCF^{SKP2} and another UPS, anaphase-promoting complex/cyclosome (APC/C) FZR1 E3 ligase (also known as CDH1) control each other, with SKP2 being ubiquitinated by APC/C^{FZR1} in G_1 cell cycle [12]. In

agreement with this, we demonstrated that GTN-stabilized nuclear CDKN1B protein level in the Huh-7 cellular model by targeting SKP2 degradation. On the other hand, MG132 treatment indicated that SKP2 itself was subjected to UPS regulation. However, the protein level of E3 ligase of SKP2, FZR1 was unchanged following GTN treatments in time-course experiments, suggesting that GTN-targeted SKP2 degradation might be mediated by another E3 ligase.

In the Hep-3B cellular model, both *CDKN1C* mRNA and protein levels were upregulated by GTN. Although SCF^{SKP2} was also reported to target CDKN1C by ubiquitin-mediated degradation [24], GTN did not significantly alter the SKP2 protein levels in a time-course experiment. These observations suggested that GTN enhanced *CDKN1C* transcription and its subsequent translation, i.e., GTN-induces *CDKN1C* transcription epigenetically [42]. Histone modifications such as hyperacetylation of histone H3 and H4 are known as active marks and are often associated with ongoing transcription [51]. We showed that treatments with an HDAC inhibitor (HDACi) such as TSA rather than an inhibitor of DNA methyltransferase such as 5-Aza-dC, significantly upregulated the *CDKN1C* mRNA levels in Hep-3B cells. Besides, GTN and TSA induced *CDKN1C* transcription synergistically. The TSA-specific inhibitor, ITSA1 downregulated the endogenous as well as GTN-induced *CDKN1C* mRNA levels strongly advocating that GTN is an HDACi that partially exhibits similar function as TSA. To our surprise, when cotreatment with TSA and GTN, or GTN and ITSA1, *CDKN1C* mRNA levels did not immediately reflect to their corresponding protein levels, i.e., delayed translation was observed. In addition, GTN and TSA additively

induced global acetyl-histone H3 but not H4 levels. Importantly, both TSA and GTN can induce co-occurrence of H3K9 and H3K14 acetylation. These results strongly suggested that GTN acts as a histone H3-specific HDACi to activate *CDKN1C* transcription and its subsequent translation.

Intriguingly, GTN targeted the degradation of SKP2, an oncoprotein in human hepatocellular carcinoma [7], in Huh-7 but not in Hep-3B cells. A potential explanation for this is the diverse genetic backgrounds between these cell lines, since Hep-3B cell line lack TP53 and RB1 proteins. It has been shown that GTN (50 μM, 10 min) directly affected mitochondria leading to a hypoxia condition in coronary artery smooth muscle cells [8]. In pulmonary artery smooth muscle cellular model from Sprague-Dawley rats and pulmonary arteries in hypoxia-induced pulmonary hypertension rats, hypoxia induces *SKP2* mRNA and then protein levels [31]. Instead, hypoxia induces *CDKN1B* mRNA expression increases with hypoxia but does not prolong *CDKN1B* mRNA half-life in Rat1a fibroblasts [13]. Accordingly, there is no direct evidence that GTN-induced hypoxia targeted SKP2 degradation. On the other hand, in human proximal tubular epithelial HK-2 cells, *CDKN1C* mRNA was upregulated at early time points of hypoxia, indicating it is within the early wave of hypoxia-responsive gene [30]. Accumulating evidence has indicated that hypoxic microenvironment is one of the most important cellular stimuli in terms of the regulation of cellular epigenetic status via histone modification (review in [38]). Thus, GTN might upregulate *CDKN1C* mRNA by hypoxia-induced epigenetic modification, particularly in TP53- and RB1-negative cells. Alternatively, GTN might potentially induce epigenetic modifications in Huh-7 cells that indirectly trigger SKP2 degradation.

We also demonstrated that GTN suppressed the protein levels of several positive cell cycle regulators, including CKS1B, CCNE1, CCND1, CDK2, CDK4, CDK6, E2F1, and/or TFDP1, involved in G_1/S transition of the cell cycle in Huh-7 and Hep-3B cell lines. During the cell cycle progression, D-type cyclins are synthesized first and form complexes with CDK4 or CDK6, which then move into the nucleus in eukaryotes. These complexes actuate the activation of later-acting CDK-cyclin complexes in two ways. First, CDK4-cyclin D-mediated phosphorylation inactivates the RB family proteins, allowing the E2F-mediated transcription of the genes encoding E-, A-, and B-type cyclins. Next, CDK4-CCND1 sequesters inhibitory proteins such as CDKN1B and CDKN1C that would otherwise impede the activity of E-, and A-type cyclin-bound CDKs (reviewed in [35]). Consistent with these findings, GTN upregulates the protein levels of CDKN1B and CDKN1C; downregulates CCKD1, CCNE1, and other positive cell cycle regulators.

Thus, we discovered that GTN is able to upregulate the protein levels of two critical CKIs, CDKN1B and CDKN1C in distinct HCC-derived cells. In TP53-mutated and RB1-positive Huh-7 cells, GTN targets the E3 ligase SKP2 degradation and stabilizes CDKN1B protein level. On the other hand, in TP53- and RB1-negative Hep-3B cells, GTN acts as an HDACi, triggers global acetylation on histone H3, including H3K9/14, thereby inducing *CDKN1C* transcription and its subsequent translation. Our findings provide novel indications for HCC management.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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