Acta Poloniae Pharmaceutica – Drug Research, Vol. 77 No. 2 pp. 295–304, 2020
DOI: 10.32383/appdr/118961
Received 28 January 2020, Received in revised form 12 February 2020, Accepted 13 March 2020.

ISSN 2353-5288
Polish Pharmaceutical Society

DRUG BIOCHEMISTRY

CAMPTOTHECIN INHIBITS MIGRATION, INVASION AND CLONOGENIC PROPERTY OF LIVER CANCER CELLS BY MODULATING MICRORNA EXPRESSION

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Abstract: Camptothecin (CPT), an alkaloid natural product, extracted from Camptotheca *acuminata* bark, has been reported to have potential antitumor activity in diverse cancers. MicroRNAs (MiRNAs) are a class of short, non-coding RNAs that plays a crucial role in the normal physiology by attenuating translation. Here, in this study, we showed that the CPT modulates the expression of miRNAs in hepatocellular carcinoma cells (HCC) Huh7. Microarray analysis reveals that CPT modulates the expression of as many as 39 miRNAs in HCC cells (Huh7), 27 miRNAs were downregulated whereas 12 miRNAs were upregulated. miR-16 is the key miRNA upregulated by CPT and targets key prosurvival proteins (MMP-2, MMP-9 and cyclin D1). Our results demonstrate that CPT is inhibiting cell viability of HCC cells significantly when compared with the untreated cells. Wound healing and colony formation assay confirm inhibition of cell migration and clonogenic property of Huh7 cells respectively, upon the dose-dependent treatment of CPT. Furthermore, the Boyden chamber assay analysis revealed significant inhibition of a number of invasive cells in CPT treated cells with comparison to untreated Huh7 cells. Mechanistically, CPT upregulates miR-16 expression which targets MMP-2, MMP-9, cyclin D1 downregulation and subsequently upregulates the expression of E-cadherin, TIMP1, p21, and p27, thereby inhibits cell migration, invasion and clonogenic property of HCC cells. In summary, CPT treatment in Huh7 cells decreases cell viability and upregulates miR-16 expression, which results in inhibition of cell migration, invasion and clonogenic property of cells, by decreasing MMP-2, MMP-9, cyclin D1 and increasing the expression of cell cycle-regulated proteins p21 and p27.

Keywords: Camptothecin, hepatocellular carcinoma, miRNA, cell cycle, cell proliferation

MiRNAs are small intracellular, noncoding RNA molecules that regulate gene expression both at posttranslational as well as a posttranscriptional level (1). Generally, they are 18-22 nucleotide long RNA molecules which induce translational attenuation by binding with the 3'-UTRs of target mRNA via imperfect matching, thereby causing messenger RNA (mRNA) degradation results in inhibition of protein translation (2, 3). MiRNAs can regulate the multi-gene expression individually or in combination with other miRNAs (4). To date, more than 2500 miRNAs have been identified in humans, and miRNA conserved targets regulate around one-third of all human genes (5). Recent studies have documented that miRNAs are not only involved in the developmental processes such as cell differentiation but also play a crucial role in the pathophysiology of tumorigenesis and has been reported on the aberrant expression of miRNA profile in cancer patients compared to normal patients (6, 7). Genomic analysis reveals that genes encoding miRNAs are commonly found at those sites in the genome where breakpoints or amplification, loss of heterozygosity are frequently occurring (8-10). Further analysis revealed that some miRNAs are downregulated or upregulated during tumorigenesis. The miRNAs which are downregulated are called tumor suppressor miRNAs and the miRNAs which are upregulated are called oncogenic miRNAs or oncomirs. For example, in lung cancer, the downregulation of let-7 results in the upregulation of Ras oncogene (11). In a Similar study, miR-122a downregulation in

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HCC regulates cyclin G1 (12). Moreover, miRNA has been reported to be modulated by chemotherapeutic drugs (13). Due to the modulations of key miRNAs or miRNA cluster by the antitumor agents, makes them a promising target player in the cancer diagnostics and therapeutics (14-16).

CPT is an alkaloid class of natural compound and its analogs are well-known antitumor agents against diverse malignancies including blood associated malignancies and solid tumors (17). The major antitumor mechanism of these chemotherapeutic compounds is activation and induction of programmed cell death type I (apoptosis) (18). Undeniably, in recent reports from various scientific laboratories revealed that CPT persuades tumor cell killing by activating apoptosis (19, 20). Besides, the induction of apoptosis, recent reports suggest that CPT and its novel derivatives have been reported to inhibit cell migration and invasion of cancer cells which are the key events in the cancer metastasis (21-23). However, it is yet to be elucidated completely the underlying mechanism of inhibition of cell migration induced by CPT. Intriguingly, recent reports point out that antitumor agent's influence in both chemoprevention and tumorigenesis by modulating key miRNAs or cluster of miRNAs. In the current study, microarray analysis demonstrates that CPT modulates the expression of numerous miRNAs in HCC cells (Huh7). Additionally, we found CPT inhibits cell viability, upregulates miR-16 expression which results in inhibition of cell migration, invasion and clonogenic property of cells, by decreasing cyclin D1, MMP-2, MMP-9 and increasing the expression of cell cycle-regulated proteins p21, p27, TIMP1, and E-cadherin in Huh7 cells.

MATERIAL AND METHODS

Cell culture and treatments

HCC cells (Huh7 and HepG2) were procured from the American Type Culture Collection (ATCC). The cells were cultured in 5% CO_2 humidified incubator and maintained the cells in Minimal Essential Medium (MEM) (#31095-029), supplemented with 10% fetal bovine serum (FBS) (#16000044), and 1% penicillin-streptomycin solution (#15140130). Cells were periodically checked free from all contaminations including Mycoplasma.

Preparation of CPT

20 mM solution was prepared from CPT powder in dimethyl sulfoxide (DMSO) as a stock solution. The stock solution was aliquoted in 1.5 mL micro-centrifuge tubes and store at -20°C. The desired working dilutions were prepared in culture media between 10 nM and 10 µM for the treatment of HCC cells (Huh7 and HepG2).

Chemicals, reagents, and antibodies

CPT (#208925), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (#M5655), propidium iodide (PI) (#P4170), phenylmethylsulphonyl fluoride (PMSF) (#78830), and DMSO (#C6164) were purchased from Sigma. However, all antibodies were procured from cell signaling technology is β-actin (#3700S, 1:10000 dilution), cyclin D1 (#55506, 1:1000 dilution), p27 (#3686, 1:1000 dilution), p21 (#2947, 1:1000 dilution), TIMP1 (#8946S, 1:1000 dilution), MMP-2 (#40994S, 1:1000 dilution), E-cadherin (#14472S, 1:2000 dilution) and MMP-9 (#13667S, 1:1000 dilution). The secondary anti-mouse IgG (#sc-2005, 1:2000 dilution) and anti-rabbit IgG-tagged (#sc-2357, 1:2000 dilution) with horseradish peroxidase (HRP) antibodies were ordered and purchased from Santacruz Biotechnology.

Cell viability assay

The proliferation of cells was analyzed by MTT assay as per the standard protocol (24). Briefly, HCC cells (Huh7 and HepG2) were processed for trypsinization and plated at a density of 5×10^3 cells per well of 96-well plate. The cells seeded in triplicates were treated with varying concentrations of CPT (100 nM – 10 µM) and control DMSO for 24 h, incubated in a humidified incubator containing 5% CO2. Subsequently, cells were saturated with MTT dye (2.5 mg/mL) for 3 h at 37°C. The crystals formed of formazan were solubilized in DMSO, mixed properly by vortex and the optical density measured at 570 nm by multi-plate reader. The percentage of cell proliferation was analyzed as the percent cell viability of treated cells compared with the control of DMSO cells.

Scratch motility (wound healing) assay

Briefly, HCC cells (Huh7) were trypsinized and distributed at a concentration of 5.5×10^5 cells per well of 6-well plate and let them adhered to make a complete monolayer for 24 h. The monolayer formed was scratched with a sterile micropipette tip (200 µL) to create a wound and were gently washed with medium without serum to washout detached cells and photographs were captured at the time of creating wound (0 h). Cells were incubated in low serum media (1%) and simultaneously exposed to varying concentrations of CPT (0.25, 0.50, 1.0 and 2.0 µM) along with DMSO for 24 h.

The photographs were captured with an inbuilt camera of the microscope from the areas where wounds were created initially after the completion of treatment time. The closure of the wound was determined as a percentage by using formula.
Closure wound %age = [1-(area of wound after 24 h/ area of wound at 0 h) × 100%]

Matrigel invasion assay

To evaluate the anti-invasive potential of CPT, Transwell Boyden chamber plates obtained from BD bioscience were used. HCC cells (1.25×10^6) were trypsinized, seeded and incubated overnight to get adhered and exposed to varying concentrations of CPT (0.25, 0.50, 1.0 and 2.0 µM) along with untreated as a vehicle for 24 h in medium without serum in the upper chamber or well and the lower chamber or well was filled with 10% complete medium as a chemoattractant. After the completion of 24 h, the Matrigel-coated polycarbonate biological porous membrane inserts were removed and the cells attached from the upper side of the chamber were scrapped completely with a swab of cotton. The porous inserts containing adhered cells from the lower chamber side were fixed in ice-cold methanol for 10 min and stained by flooded with 0.1% crystal violet solution. The migration of the cells was analyzed by capturing photographs and counted the migrated stained cells under the phase-contrast microscope.

Colony formation assay

The assay was used to evaluate the clonogenic property of cells. Briefly, 1×10^3 Huh7 cells were seeded in a 6-well plate and let them attach overnight in a humidified incubator at 37°C. Next morning, change the media with fresh media and were treated with varying concentrations (0.25, 0.50, 1.0 and 2.0 µM) of CPT for 24 h along with control DMSO for 5 days at 37°C in 5% CO_2 incubator. After the completion of time point, each well is fixed with ice-cold methanol followed by staining with 0.5% crystal violet solution. The colonies stained in each well were counted and photographed with an Olympus c-7070 microscope with an inbuilt microscope.

Immunoblotting

After overnight plating of 50×10^5 cells/well in humidified 5% CO_2 incubator at 37°C, HCC cells were exposed with varying concentrations (0.50, 1.0, 2.0 and 4.0 µM) of CPT along with DMSO vehicle. After 24 h, cells were harvested and cleaned in PBS which was earlier stored at 4°C. The cells were processed for lysis with cell lysis buffer. The lysis solution obtained from cells was collected and processed at 4°C for centrifugation at a speed of 13000 g for 10 min. Supernatants collected from centrifugation in a separate tube were subjected to the Bradford method of protein estimation. The 20 µg of protein concentration was calculated from each treated sample and were loaded in the wells of the gel formed of SDS-PAGE. After resolving properly, SDS-PAGE gel was subjected to transfer onto the PVDF membrane, allowed it to incubate in fat-free milk (5%) blocking solution dissolved in TBST. After blocking nonspecific proteins with a blocking solution, the PVDF membrane is incubated and probed with the desired antibodies (1 : 1000 dilutions) for at least 3-4 h when incubated at room temperature or 4°C overnight. The PVDF membrane is gently rinsed with TBST buffer three times. The pre-incubated membrane with the primary antibody is probed with a horseradish peroxidase-labeled secondary antibody. Immunosubstance were spotted by enhanced chemiluminescence (ECL) plus

MiRNA microarray analysis

Total RNA was extracted by mirVana RNA isolation kit, after treating HCC cells (Huh7) with 2 µM CPT in 100 mm culture dishes for 24 h. 50 µg of total RNA was enriched for small species of RNA, and 3' amine-tailed modification was performed by mirVana miRNA tagged kit and fluorescent-tagged with amine-reactive Cy3 (control untreated cells) or Cy5 dyes (2 µM CPT treated cells). Subsequently, the co-hybridization of RNAs tagged with fluorescent dyes at 42°C for 14 h on mirVana miRNA Bioarray. As per manufacturer protocol, the microarray was washed and the fluorescence was scanned by GenePix scanner.

Real-time polymerase chain reaction (RT-PCR)

To evaluate the miRNA expression attained from microarray, using Trizol reagent (Invitrogen, Carlsbad, CA), total RNA from cells (untreated and treated with CPT) was extracted. RNA (3 µg) obtained from extraction, was reverse transcribed using the QuantMir RT Kit. Using an ABI 7900HT Sequence Detection System (ABI Applied Biosystems, Foster City, CA), Real-time PCR was performed by adding Syber-Green PCR Master Mix. For the detection of each miRNA, the DNA sequence of mature mRNA was used as a forward primer and the reverse primer called 3' universal primer was provided by QuantiMir RT Kit. The PCR reaction was run in a 96- well plate for 10 min at 95°C followed by 40 cycles of 95°C for 15 sec and 50°C for 1 min. ΔC_t was measured by dividing the C_t of U6 small nuclear RNA from C_t of the miRNA of

interest. $\Delta\Delta C_t$ was then measured by dividing the ΔC_t of the vehicle from the ΔC_t of the CPT treated cells. The expression of miRNA analysis was performed by using the comparative threshold cycle (2-ΔΔCT) method.

Transient transfection with miR-16 precursor

Briefly, 50×10^5 Huh7 cells were transiently transfected with miR-16 precursor using lipofectamine 2000 R for 36 h followed by treatment with varying concentrations of CPT for the next 24 h. Further, cells were subjected to cell lysis for protein determination and immunoblotting.

Statistical analysis

All the evaluated experiments were accomplished for equal or more than three independent times. The latest version of software GraphPad Prism was used for statistical analysis of all independent unbiased experiments. The results obtained were denoted as the mean of ±SEM. Entire results were calculated by using the Student's unpaired t-test, wherein a p-value not more than 0.05 was reflected significant (* means $p < 0.05$. ** $p < 0.01$, and *** $p < 0.001$).

RESULTS

CPT inhibits cell proliferation, migration, invasion, and clonogenic property of HCC cells

In the recent past, numerous reports demonstrated that CPT exhibits an antiproliferative effect on various types of tumor cell lines. Hence, we wanted to inspect the *in vitro* inhibitory activity of

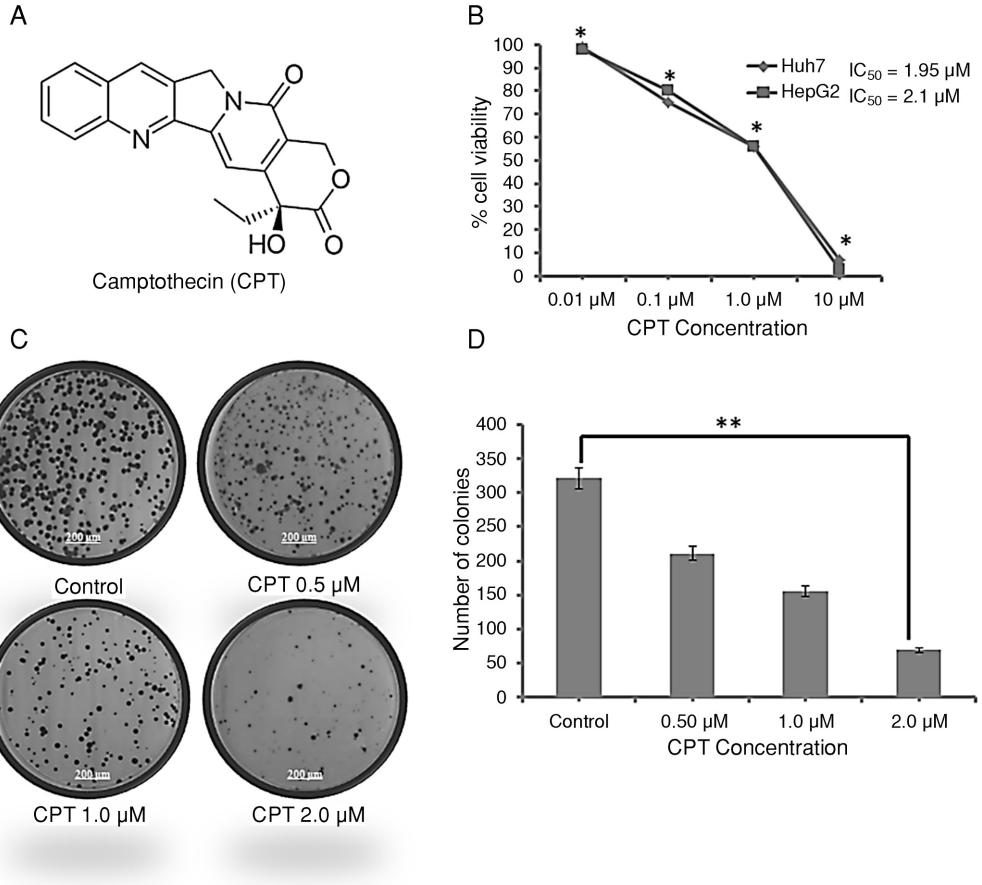

Figure 1. CPT inhibits cell proliferation and clonogenic properties of HCC cells. (A) Chemical structure of Camptothecin (CPT). (B) The effect of CPT on the cell viability of HCC (HepG2 and Huh7) was determined by the MTT assay. (C) Huh7 cells were treated with CPT in concentration-dependent manner along with positive control staurosporine and untreated control for 24 h, to determine the anti-clonogenic property of CPT. Scale bar: 200 µm; 10x (D) Bar diagram showing quantification of a number of colonies of Huh7 cells per field. The data represent the mean value ±SE of three independent experiments *$p < 0.05$, **$p < 0.01$.

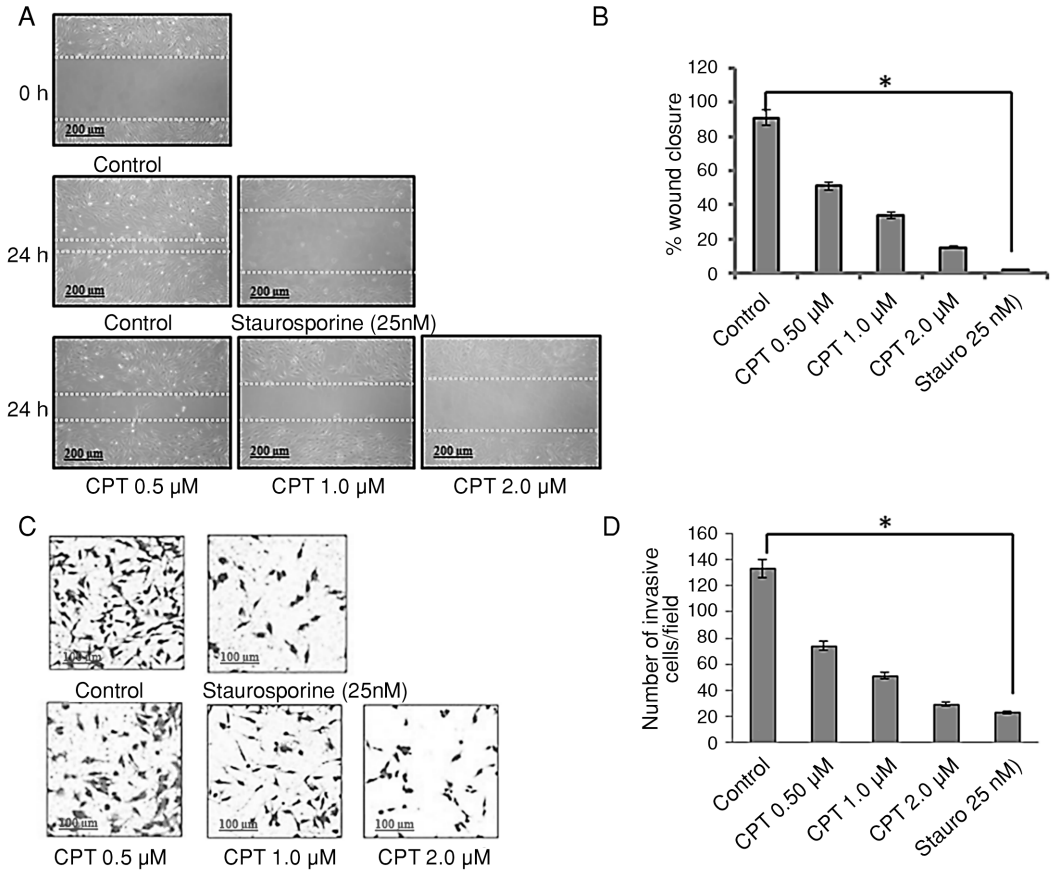

Figure 2. CPT inhibits cell migration and invasion of Huh7 cells in a dose-dependent manner. (A) Huh7 cells were exposed to indicated concentrations of CPT (0.5, 1.0 and 2.0 µM along with staurosporine as positive control and DMSO as a control) for 24 h to evaluate the anti-motility effect. Scale bar: 200 µm; 10x (B) Bar diagram showing the percentage of wound closure with Huh7 cells after exposure with varying concentrations of CPT. (C) Cell invasion was determined by the Transwell Boyden chamber assay. Briefly, 2×10^5 cells/well was seeded in the top chamber in the presence of CPT along with control DMSO. Cells were allowed to migrate for 24 h, cells able to cross the porous membrane from the bottom half of the insert membrane were stained with 0.1% crystal violet and counted under phase-contrast microscope. Five fields were counted in triplicate from each insert of three independent experiments. Scale bar: 100 µm; 20x (D) Bar diagram showing quantification of invasive cells after exposure with varying concentrations of CPT. The data represent the mean value ±SE of three independent experiments *$p < 0.05$.

CPT on the growth of HCC (Huh7 and HepG2) cells. The CPT inhibits HCC cells in a concentration-dependent manner. The MTT assay performed to analyze the inhibitory activity on cell viability revealed that CPT initiated an antiproliferative effect on both the types of HCC cells (Huh7 and HepG2) at a concentration of 0.5 µM. However, when calculated the IC50 concentration values of CPT by GraphPad prism software, we found that the IC50 value is 1.95 µM and 2.1 µM on Huh7 and HepG2 cells respectively (Fig. 1B). These results revealed that CPT exhibits promising antiproliferative effect on HCC cells

Besides antiproliferative activity, we also intended to evaluate whether the colony formation ability of HCC cells was also inhibited by CPT treatment. As shown in Figure 1C and 1D that subtoxic concentrations of CPT significantly attenuate the clonogenic property of Huh7 cells.

To examine whether, CPT could also impact tumor cell motility, migration, and invasion *in vitro*. We intended to perform wound healing assay and Transwell Boyden chamber cell migration assay *in vitro* to evaluate the anti-migration or anti-invasive potential of CPT by treating cells with subtoxic concentrations of CPT in HCC cells. Due to the significant induction of cell death at higher concentrations of CPT, sub-toxic concentrations (0.50 1.0 and 2.0 µM) of CPT for 24 h were carefully selected to study the anti-motility and anti-invasive effect on HCC cells. Our results revealed that a significant reduction in motility of cells was witnessed once HCC

Table 1. Modulation of miRNA expression profile after CPT treatment in Huh7 cells for 24 h.

microRNA	Map	Normalization mean ratio CPT/control	*p*-Value	Up-/down regulation
let-7a	9q22.2	2.1	0.0134	Up
Iet-7b	22q13.3	1.9	0.0467	Up
let-7c	21q11.2	2.5	0.0497	Up
let-7d	9q22.2	2.4	0.0012	Up
miR-10a	17q21.32	-3.1	0.0154	Down
miR-16	8q24.22	3.1	0.0386	Up
miR-18a	13q31.3	-2.1	0.0287	Down
miR-18b	Xq26.2	2.9	0.0243	Up
miR-19a	13q31.3	-1.9	0.0398	Down
miR-20a	13q31.3	1.97	0.0321	Up
miR-25	7q22.1	1.99	0.0432	Up
miR-26b	2q35	-2.13	0.001	Down
miR-29b	7q32.3	-2.8	0.05	Down
miR-34b	11q23.1	-2.54	0.0489	Down
miR-92	13q31	2.65	0.0451	Up
miR-93	7q22.1	2.1	0.0428	Up
miR-98	Xp11.22	-1.67	0.0359	Down
miR-99b	19q13.4	-1.98	0.0411	Down
miR-129	7q32.1	-1.72	0.0416	Down
miR-181d	19p13.12	-1.87	0.001	Down
miR-182	7q32.2	-1.98	0.01	Down
miR-186	1p31.1	-3.12	0.0349	Down
miR-193b	16q13.12	-2.13	0.0499	Down
miR-196a	17q21.32	-3.11	0.0418	Down
miR-196b	7p15.2	-2.98	0.0421	Down
miR-199a	19p13.2	-2.78	0.0451	Down
miR-200a	1p36.33	-1.76	0.0395	Down
miR-205	1q32.2	-1.67	0.0421	Down
miR-210	11p15.5	-5.76	0.0331	Down
miR-217	2p16.1	-3.89	0.0445	Down
miR-221	Xp11.3	3.65	0.0233	Up
miR-222	Xp11.22	-1.67	0.0251	Down
miR-320	8p21.3	1.99	0.0443	Up
miR-376a	14q32.31	-3.67	0.0344	Down
miR-497	17p13.1	-2.98	0.0481	Down
miR-507	Xq27.3	-4.78	0.0452	Down
miR-516	19q13.42	-1.67	0.0122	Down
miR-517c	19q13.42	-1.87	0.0493	Down
miR-519	19q13.42	-1.99	0.009	Down

cells (Huh7 and HepG2) were exposed to 1.0 and 2.0 µM of CPT, which as good as positive control staurosporine (25 nM) (Fig. 2A and 2B), after creating a wound by making a scratch with 100 µL sterile tip when compared to a control DMSO that was almost filled with migrated cells.

A crucial step of metastatic cells is the capability of these cells to migrate across the various barriers including extracellular matrix and disseminate the migrated cells away from the primary tumor site. To study the influence of CPT on the invasive property of cancer cells, we intended to perform a Transwell Boyden chamber assay to determine the inhibitory capability of CPT on HCC cell migration and invasion through the porous biological membrane inserts *in vitro*. Our Transwell Boyden chamber assay results demonstrate the significant number of invaded cells lose the ability to penetrate through the matrigel coated membrane in a dose-dependent manner when compared to control DMSO (Fig. 2C). The bar diagram (Fig. 2D) showed that the number of invaded cells is 74, 51, and 29 at a concentration of 0.5, 1.0, and 2.0 µM of CPT respectively, as compared to 133 invaded cells in control DMSO. Collectively, these results indicate that subtoxic doses of CPT attenuate the growth kinetics of HCC cells.

CPT modulates miRNA expression of Huh7 cells

Recent reports demonstrate that various natural products modulate miRNA expression and inhibit cell viability in cancer cells. Therefore, we tend to seek whether, CPT mediated inhibition of cell viability, migration, and invasion (above-mentioned data) could also modulate the expression of miRNAs in Huh7 cells. To investigate whether CPT could modulate the expression of miRNAs, Huh7 cells were exposed to 4 µM of CPT for 24 h. After the completion of treatment, total RNA was isolated to analyze miRNA by microarray. The differential expression of CPT treated cells and control DMSO cells were observed by applying a filter based fold change expression of more than 1.5 and statistically significant. As shown in Figure 3A, 3B and Table 1, in a microarray of 328 human miRNAs, 27 miRNAs were downregulated and 12 were upregulated when Huh7 cells were exposed to 4 µM of CPT treatment. Additionally, to corroborate the finding obtained after

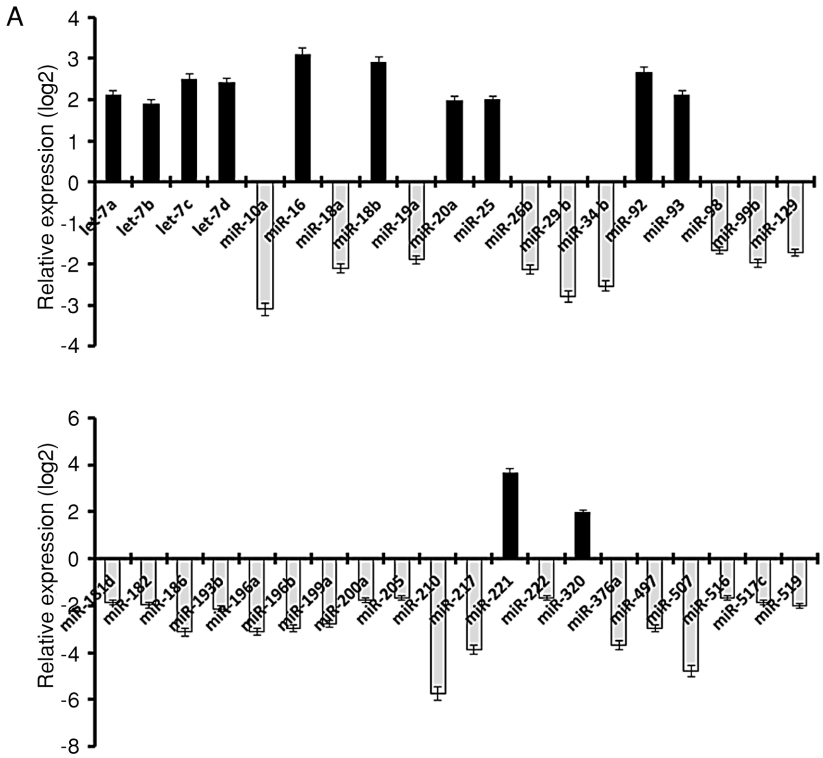

Figure 3. CPT modulates miRNA expression profile in Huh7 cells. (A) Huh7 cells were exposed to 4 µM concentration of CPT for 24 h. The data represent the mean value ±SE for the fold change in the expression of miRNA in log 2 scale between CPT-treated Huh7 cells and untreated Huh7 cells obtained from three independent experiments.

microarray analysis, four miRNAs expressed differentially (upregulated miR-16, and let-7a; downregulated miR-34b, and miR-222) were evaluated by real-time quantitative PCR. The relative miRNA expression of Huh7 cells treated with CPT and control DMSO treated cells were observed to be similar to the results obtained by microarray analysis.

MiR-16 mediated CPT induction of cell cycle checkpoint proteins and downregulates cell survival and metastatic proteins in Huh7 cells

Recent reports demonstrated that MMPs and cell cycle proteins play a critical role in invasion and metastasis of tumor cells. Intriguingly, miRNAs are the novel players which also regulates proteins both at transcriptional as well as translation level. In previous results, we found CPT treatment modulates miRNA expression in Huh cells. Among them, miR-16 is a key tumor suppressor miRNA upregulated in CPT treatment. Therefore, we intended to explore whether increased expression of miR-16 mediated by CPT could inhibit the survival proteins in HCC cells. As shown in Figure 4A, 4B, the immunoblotting results of HCC cell lysates initially transfected with miR-16 followed by dose-dependent concentration (2.0, 4.0 μM) treatment with CPT inhibits protein expression of cyclin D1, MMP-2, and MMP-9 with concomitant induction in the expression of E-cadherin, p21, p27, and TIMP1. Collectively these results demonstrate that miR-16 mediated CPT attenuates cell migration by downregulating proteins cyclin D1, MMP-2, MMP-9 and upregulating the expression of p21, p27, and E-cadherin in Huh7 cells.

DISCUSSION

In the current study, we evaluated the inhibitory role of CPT in cell migration, invasion, clonogenic property as well as modulation of miRNAs in HCC (Huh7) cells. We demonstrated that tumor suppressor miR-16 expression is upregulated in CPT treated Huh7 cells and downregulated many oncomirs. We showed miR-16 mediated CPT downregulates the expression of cell survival proteins and upregulates the expression cell cycle check proteins p21, p27 with subsequent induction of TIMP1 and E-cadherin expression in Huh7 cells. These results demonstrated that the induction of miR-16 by CPT is a novel mechanism for cancer therapeutics in the future.

In the recent past, CPT has gained tremendous attention as a promising chemotherapeutic agent. Numerous studies demonstrated the anti-cancer effect of CPT against a wide variety of human cancer cells (25). Moreover, CPT exerts anti-tumor activity by modulating a plethora of molecules that play a crucial role in the development of tumorigenesis (26). A growing body of evidence revealed that CPT exerts antiproliferative effect by inducing mitochondrial-dependent apoptosis in cervical cancer cells by influencing miR-125b (27). Consistent with

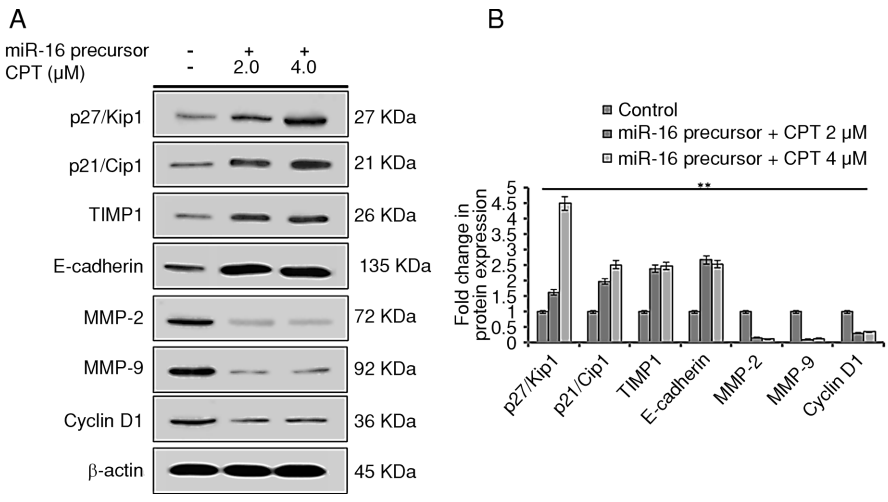

Figure 4. MiR-16 mediated CPT induction of cell cycle checkpoint proteins and downregulates cell survival and metastatic proteins in Huh7 cells. (A) Huh7 cells transiently transfected with miR-16 precursor followed by CPT treatment (2 and 4 μM) for 24 h along with control DMSO. Cells were processed for cell lysis and immunoblotting and probed with cell cycle regulatory proteins (p27, p21), metastasis-associated proteins (MMP-2, MMP-9), and TIMP1 and E-cadherin proteins along β-actin as an internal control. (B) Densitometry analysis represented by histogram shows the fold change in protein expression after normalized with β-actin. The data denotes the mean value ±SE of three independent unbiased experiments **$p < 0.01$.

these results, we notice that CPT exerts antiproliferative effect by inhibiting cell migration, invasion and clonogenic property of Huh7 cells. Our results revealed that CPT displays a strong antiproliferative effect by reducing cell viability of HCC cells significantly with IC_{50} of 1.95 and 2.1 μM in Huh7 and HepG2 cells respectively.

Yang et al. showed that in breast cancer cells, a novel analogs of CPT (FL118) suppresses the invasive as well as motility of tumor cells by impairs the Wnt/β-catenin signaling pathway thereby inhibits epithelial to mesenchymal transition (28). Consistent with previous studies, we observe CPT attenuates colony formation ability and exerts a significant reduction in motility of Huh7 cells. To discourse any rational discrimination of CPT in modulating cell migration with apoptosis, we cautiously select subtoxic concentrations and found that 1.0 μM CPT altered cell migration significantly even at subtoxic doses when compared to untreated cells which were filled with migrated cells. A crucial step of metastatic cells is the capability of these cells to migrate across the various barriers including extracellular matrix and disseminate the migrated cells away from the primary tumor site (29). Interestingly, the Transwell Boyden chamber invasion assay also supports the notion that subtoxic concentrations of CPT significantly inhibit many invaded cells to penetrate through a porous biological membrane.

Recent advancement in genomics demonstrates that miRNAs are key players in the development of tumorigenesis (30). The modulation of miRNAs by antitumor agents plays a crucial role in cancer therapeutics. As evidenced by microarray analysis of Huh7 cells exposed to varying concentrations of CPT modulates as many as 39 miRNAs compared to untreated cells. Among the miRNAs modulated by CPT, some are known to target oncogenic activity. MiR-16 and let-7 are the miRNAs that act as tumor suppressor miRNAs and are upregulated by CPT treatment in Huh7 cells. MiR-16 is known to attenuate antiapoptotic protein Bcl-2, whereas let-7 has a role in suppression of Ras activity in cancer cells. The miRNAs which are downregulated by CPT treatment are oncomirs miR-34b and miR-222. These oncogenic miRNAs play a very important role in the antiapoptosis mechanism. Collectively these findings reveal that CPT modulates biological activities by upregulating and downregulating the expression of miRNAs.

Accumulating evidence demonstrated that miR-16 mediates activation and induction of apoptosis by inhibits antiapoptotic protein Bcl-2 in a variety of tumor cell lines. Moreover, recent reports suggest the strong correlation between increased expression of miR-16 by epigallocatechin (EGCG) and reduction in Bcl-2 expression (31). In our study, we found that CPT treated Huh7 cells upregulates miR-16 expression when compared to untreated cells. Additionally, to evaluate whether miR-16 has any role in suppression of cell survival proteins which play a crucial role in cell cycle and metastasis, we transfect cells with miR-16 initially and exposed the cells with increasing concentrations of CPT. Interestingly, our results demonstrated that a drastic decrease in the expression of Bcl-2 as well as cell survival proteins cyclin D1, MMP-2 and MMP-9 with subsequent upregulation of cell cycle checkpoint proteins p21, p27 and TIMP1 as well as E-cadherin expression in Huh7 cells. Taken together these results demonstrate that miR-16 mediated CPT blocks cell migration and invasion of HCC cells.

CONCLUSION

Our results first time demonstrate that CPT exerts antiproliferative effect, and abrogates cell motility and invasion of HCC cells by modulating the expression of miRNAs. Additionally, we found miR-16 mediated CPT decreases cyclin D1, MMP-2, and MMP-9 with subsequent induction of cell cycle-regulated proteins p21, p27, TIMP1, and E-cadherin in Huh7 cells. Finally, we demonstrate that CPT is a promising anticancer therapeutic agent against HCC and could be used in future therapeutics for the prevention and therapeutics of HCC.

Acknowledgments

The authors would like to thank the Department of General Surgery, The Second Affiliated Hospital of Shandong First Medical University, Taishan Street, Tai'an, Shandong, China for grant and support.

Authors contributions

Song Wu: Conceptualization, Visualization Data curation, acquisition, Formal analysis. **Xiaoqian Li:** Conceptualization, Writing, review & editing. **Zhenzhong Liu:** Conceptualization, design experiments, writing review and editing the original draft.

Conflict of interest

None

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