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Original article

Cisatracurium-induced proliferation impairment and death of colorectal cancer cells, HCT116 is mediated by p53 dependent intrinsic apoptotic pathway in vitro

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ABSTRACT

Activation of oncogenes and suppression of repressor genes are believed to play crucial roles in the pathogenesis of human colorectal carcinoma. Cisatracurium, a nondepolarizing neuromuscular blocking agent, has been reported to inhibit cell proliferation while promoting apoptosis. However, the underlining mechanism, of these growth setbacks are not well understood. We assessed the growth of human colorectal carcinoma (HCT116) and its cell cycle distribution upon cisatracurium exposure. Significant cell growth inhibition and accumulation of cells in G1 phase of the cell cycle was observed in treated cells compared with untreated cells (control). In furtherance to these observations, FITC Annexin V and propidium iodide apoptosis assay demonstrated concentration and time dependent percentage increase in apoptosis of cells treated with cisatracurium compared with untreated cells. qRT-PCR analysis showed concentration-dependent alterations in CD1, E2F, CE1, p53 and p21 mRNA expression. Western blot analysis indicated remarkable concentration dependent alterations in the expression of proliferation and survival proteins CD1, E2F, CE1, p53, p21, BAX, BCL-2, cytochrome C and cleaved PARP in cisatracurium-treated groups as compared with the untreated group. Cisatracurium also significantly promoted caspase-9 and caspase-3 activities in cells treated with cisatracurium compared with untreated cells. Thus, cisatracurium effectively inhibited proliferation and induced apoptosis of HCT116 cells in vitro at least via alteration of p53-dependent apoptotic pathway.

1. Introduction

More and more patients suffering from cancer receive surgery and anesthesia for cancer treatment and pain control. Therefore, a comprehensive understanding of potential and actual implications of anesthetic medications in cancer biology will remarkably improve the health of patients with cancer. One of such anesthetic drugs commonly used during surgery and occasionally in intensive care is cisatracurium. Of interest in the present study is the molecular mechanism by which cisatracurium induces growth inhibition and apoptosis in human colorectal carcinoma cells (HCT116).

Cisatracurium, a bisbenzylisoquinolinium is an isomer of atracurium which is classified as a nondepolarizing neuromuscular blocking drug [1,2]. Cisatracurium acts by competitive inhibition of nicotinic acetylcholine at the post-synaptic junction of the neuromuscular junction and subsequently prevent muscle depolarization thereby blocking neuromuscular transmission. It is eliminated from the body through spontaneous breakdown at physiologic temperature and pH (Hoffman elimination) [3,4], to form acrylate esters [5] and laudanosine [6]. As a result of this metabolic process coupled with other properties such as its intermediary duration of action, absence of histamine release and muscle fasciculation, cisatracurium is usually preferred by many anesthesiologists for smooth endotracheal intubation, mechanical ventilation and maintenance of muscle relaxation during surgery, especially in patience with respiratory difficulty [7], liver and renal impairment [8], and other abdominal disease conditions that require adequate muscle relaxation during surgery. In spite of the clinical supremacy cisatracurium has over other neuromuscular blocking agents, the uncertainty about whether this particular neuromuscular blocking agent has other deleterious clinical effect is yet to be known [9,10].

Human colorectal carcinoma is a malignant disease of human colon and rectum characterized by aberrant growth of colon cells that outgrows their normal boundaries. This type of cancer usually

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starts as a growth on the mucosal layer of the colon or rectum called a polyp, and subsequently progress if undetected early for treatment. Human colorectal carcinoma is tagged as the third most frequent among men and second in women with an estimated 1.4 million cases globally [11]. Approximately 693,900 deaths from colorectal cancer, accounting for 8% of the world's total cancer deaths, have been reported [9]. Even though the prevalence and mortality rates of colorectal cancer in America is declining [12], its prevalence and mortality rate are on the ascendancy in many developed and developing countries [13–15]. Several treatment modalities such as surgery, chemotherapy and radiation are available for colorectal cancer patients. However, surgical resection of colorectal tumors is considered as the main treatment, except in patients with wide spread metastasis. Therefore, in the event of surgical excision, the choice of anesthetic agents could defeat the purpose of the surgical intervention because many anesthetic medications such as isoflurane [16] and morphine [17] have been implicated to promote the progression of many human cancers. Surgery for colorectal cancer resection requires adequate muscle relaxation to enable easy and uneventful removal of the tumors. In such circumstance, the use of nondepolarizing muscle relaxants such as cisatracurium becomes necessary as it induces smooth and adequate muscle relaxation. Contrary to the oncopromotion property enhancement of isoflurane and morphine, it has been established that cisatracurium inhibit human cancer cells proliferation [18,19]. However, the molecular mechanism of the process is yet to elucidated. Thus, the present study was aimed to investigate the molecular mechanisms underlining cisatracurium-induced cells inhibition, and also the effect of cisatracurium on 5-fluorouracil (5-FU) cytotoxicity in human colorectal carcinoma cells (HCT116) in vitro.

2. Materials and methods

2.1. Materials

Human colorectal carcinoma cell line (HCT116) was purchased from Shanghai Institute of Cell (China). Cisatracurium was purchased from Nimbex (GlaxoSmith-Kline). 5-Flourouracil (5-FU) was purchased from Sigma–Aldrich (Steinheim, Germany). Dojindo's Cell Counting Kit-8 (CCK-8) was procured from Dojindo Molecular Technologies, Japan. Activity kits for caspase-9 and caspase-3 were obtained from Beyotime Institute of Biotechnology (Shanghai, China). Fetal bovine serum (FBS) was purchased from PAA (Australia), Rowell Park Memorial Institute Medium-1640 (RPMI-1640), 50 units/mL of penicillin and streptomycin were purchased from Gibco (USA).

2.2. Cell culture

HCT116 cells were maintained in RPMI media (with 10% FBS, 50 units/mL of penicillin and 50 units/mL of streptomycin) in CO_2 incubator under humidified atmosphere of 5% CO_2 at 37 °C. After 80% confluence, the cells were detached from the flask using trypsin. Cell pellets were collected by centrifugation at 1500 rpm for 5 min, suspended in complete media, counted and used for subsequent cell culture studies. Cell culture medium was changed two to three times a week.

2.3. CCK-8 cell proliferation assay

Cell proliferation was determined by CCK-8 assay. To determine the effect of cisatracurium on the proliferation of human colorectal carcinoma cells, 3×10^3 of HCT116 cells suspended in 100 μl of medium per well were seeded in 96-well plate and incubated for 24 h to allow cells to attach. Next the cells were treated with cisatracurium at concentrations of 10 μM or 20 μM for 24 h, 48 h and 72 h. After each treatment time point, 10 μl of CCK-8 reagent was added to untreated (control) and cisatracurium treated cells (10 μM or 20 μM). Cells were incubated for 1 h and the absorbance detected at an optical density (OD) of 450 nm using MultiskanGo Spectrophotometer (USA). Percentage inhibition was calculated using the formula: [(Average OD of untreated − Average OD of treated)/Average OD of untreated × 100].

2.4. Colony formation assay

The sensitivity of HCT116 cells to cisatracurium treatment was also assessed by clonogenic assay. In a typical setting, 300 cells were seeded into 6-well plate in triplicates, containing complete medium followed by the addition of cisatracurium (10 μM or 20 μM) to the cultures on the following day. After 48 h treatment of HCT116, the cisatracurium containing media were replaced with drug-free complete medium and further incubated for 7 days to allow colony formation. Cells were then fixed with 25% acetic acid in ethanol and stained with Giemsa. Colonies of at least 50 cells were scored. Each experiment was carried out at least three times in triplicate cultures.

2.5. Cell cycle analysis assay

To investigate the influence of cisatracurium on cell cycle progression of human colorectal carcinoma cells, HCT116 cells were synchronized by growth at 100% confluence with reduced serum for 72 h. HCT116 cells were detached using trypsin and incubated for 24 h in a complete medium. After the 24 h, medium was replaced with complete medium with/without cisatracurium (10 μM or 20 μM) for another 48 h. The cells were harvested at the log phase of growth, washed with PBS for two times in ice-cold PBS and fixed in 75% cold ethanol at 4 °C overnight. After fixation, the cells were washed twice with ice-cold PBS, 50 μg/ml of RNase (Sigma, USA) was added to the cells for 30 minutes, stained with 20 μg/ml of propidium iodide (Sigma USA) overnight in darkness and analyzed by flow cytometry (Beckman counter, USA). The data was analyzed using Multicycle software (Phoenix Flow Systems, San Diego, USA) to determine the cell cycle distributions.

2.6. Cell apoptosis analysis

The effect of cisatracurium on HCT116 cells apoptosis was investigated using Annexin V-FITC and propidium iodide apoptosis test kit (KeyGEN, China) and analyzed by flow cytometry. In summary, 1×10^6 cells were seeded into three 6 cm dishes, incubated under 5% CO_2 atmosphere at 37 °C in a humidified incubator for 24 h. After 24 h, culture medium was replaced with either complete medium only for control or complete medium with cisatracurium (10 μM or 20 μM) for treatment groups and cultured for 24 h, 48 h and 72 h. Cells were harvested at the end of each treatment time point, washed with ice-cold PBS three times, centrifuged at 1000 rpm for 5 min, resuspended in 500 μl binding buffer, incubated in 5 μl Annexin V-FITC and 5 μl PI in the dark for 30 min at room temperature and, subsequently subjected to fluorescence activated cell sorting (FACS) analysis (FACS Calibur, BD Biosciences, USA). The results were analyzed using the Cell Quest software (BD Biosciences, USA).

2.7. qRT-PCR analysis

In the in vitro experiment, total RNA was extracted from untreated cells, 10 μM and 20 μM cisatracurium-treated cells using Trizol (Invitrogen, USA) in accordance with the manufacturer's guide. Reverse transcription of purified RNA from the

untreated and cisatracurium treated samples were performed using oligonucleotide dT primer. qRT-PCR was done using SYBR green I dye and the quantification of gene transcripts was carried out and normalized to β-actin as the internal control. The pairs of primer sequences used in this work are shown in Table 1. PCR was carried out under the following conditions: 45 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C, and extension for 30 s at 72 °C. The relative mRNA expression level of untreated HCT116, 10 μM and 20 μM cisatracurium-treated HCT116 cells were analyzed using $2^{-\Delta\Delta}$CT method previously described [20]. The PCR was performed using Mx 3005P qRT-PCR machine (Agilent Technologies, Germany).

2.8. Cell lysates and immunoblotting

Protein expression in untreated and cisatracurium-treated HCT116 colon cancer cells was assessed by immunoblot technique (Western blotting) using the respective cell lysates. The cell lysates were prepared from cultures that were sub-confluent. Briefly, cells were grown to 70% confluence, treated with various concentrations of cisatracurium for 48 h, and lysed using RIPA buffer, PMSF and ×100 anti-protease cocktail for immunoblot analysis following standard protocols. The protein concentration of cell lysate was determined by BCA Protein Assay Kit using nanodrop spectrophotometer (Thermofisher Scientific, USA). Cell lysate protein (20 μg) was loaded and separated using SDS-PAGE, followed by blotting onto a polyvinylidene difluoride membrane (PVDF) (Invitrogen, USA). Proteins were detected by specific primary antibodies and their respective secondary rabbit anti-mouse (Boster, Wuhan, China, 1:5000) or mouse anti-rabbit (Boster, Wuhan, China, 1:5000) antibodies. The primary antibodies used were: anti-CD1 (Boster, Wuhan, China, 1:400), anti-E2F (Boster, Wuhan, China, 1:400), anti-CE1 (Proteintech, China, 1:500), anti-p53 (Boster, Wuhan, China, 1:500), anti-p21 (Boster, Wuhan, China, 1:400), anti-BAX (Boster, Wuhan, China, 1:400), anti-BCL-2 (Proteintech, China, 1:500), anti-cytochrome C (Boster, Wuhan, China, 1:500), anti-PARP (full length recognized at 116 kDa and fragments at 89 kDa respectively) (Proteintech, China, 1:500) and anti-β-actin (Boster, Wuhan, China, 1:500). β-actin was used as sample loading control. The bands were captured by Li-Cor Odyssey Infrared Imaging System (Version 3.0 software).

2.9. Quantification of caspase activity

We determined caspase-9 and caspase-3 activities, using Caspase Apoptosis Colorimetric Assay Kit (Beyotime Biotechnology, Shanghai, China), as directed by the manufacturer. 5×10^6 untreated HCT116 cells (control) and cisatracurium-treated HCT116 cells (10 μM and 20 μM) were seeded and lysed by incubating in 50 μl lysis buffer on ice for an hour. The lysate was thoroughly mixed using vortex. Total protein concentration from the untreated and cisatracurium-treated groups was quantified using Bradford Protein Assay Kit (Beyotime Biotechnology, Shanghai, China). Caspase activity experiment for the untreated and cisatracurium-treated groups was carried out by adding 50 μl of lysate, 50 μl of 2× reaction buffer and 5 μl of caspase substrate, and the resulting reaction mixtures were kept in an incubator at 37 °C for 4 h. Peptide degradation was quantified using a spectrophotometer with emission wavelengths set to 405 nm (T6, Beijing, China).

Table 1
Nucleotide sequence of primers used in qRT-PCR.

Gene	Primers	
	Forward	Reverse
CD1	TGTCCTACTACCGCCTCACA	CTTGGGGTCCATGTTCTGCT
E2F	ACATCACCAACGTGCTGGAA	AAACATTCCCCTGCCTACCC
CE1	TGCAGCCAAACTTGAGGAAATC	TAGTCAGGGGACTTAAACGCCA
p53	ACCTATGGAAACTACTTCCTGAAA	CTGGCATTCTGGGAGCTTCA
p21	GCGACTGTGATGCGCTAATG	GAAGGTAGAGCTTGGGCAGG

2.9.1. Cytotoxicity assay

Dojindo's CCK-8 assay was used to assess cytotoxicity. Cells were seeded into a 96-well plate (5×10^3 cells/well) in triplicates and incubated under standard growth conditions for 24 h. Cells were then treated with 5-FU (10 μM) alone and 5-FU (10 μM) with different concentrations of cisatracurium (10 μM and 20 μM) for 24 h, 48 h, and 72 h. After the respective hours of 5-FU-cisatracurium treatments, the cells were incubated with CCK-8 for 1 h. Absorbance was measured at 450 nm using MultiskanGo Spectrophotometer (USA). Percentage inhibition was calculated using the formula: [(Average OD of untreated − Average OD of treated)/Average OD of untreated × 100].

2.9.2. Statistical analysis

All the measurements were done in triplicate, and the results expressed as mean ± SEM. Statistical analysis of the data was carried out using SPSS version 17 (IBM, USA) and GraphPad Prism 6 (GraphPad Software Inc., USA). Observed differences were determined by comparative analysis using one way analysis of variance (ANOVA). Statistical significance was considered at $P < 0.05$ ($n = 3$).

3. Results

3.1. Cisatracurium retards HCT116 cells proliferation and colony formation

In a standard CCK-8 proliferation assay, the human colorectal carcinoma cells exposed to cisatracurium (10 μM or 20 μM) showed diminished growth rates in concentration and time dependent manner compared to untreated human colorectal carcinoma cells (Fig. 1A and B). In comparison with the untreated HCT116 cells, proliferation of 10 μM or 20 μM cisatracurium treated cells were inhibited by 16% and 20% on day 1; 32% and 40% on day 2 and 47% and 69% on day 3. On the bases of time, the untreated HCT116 cells increased in proliferation by 1.8% and 4.4% at 48 h and 72 h respectively compared to growth at 24 h. However, treatment with 10 μM cisatracurium inhibited proliferation by 18% and 34% at 48 h and 72 h as compared to growth at 24 h. Similar trend was observed in 20 μM cisatracurium treated HCT116 cells where proliferation decreased by 17% and 56% at 48 h and 72 h respectively as compared to growth at 24 h.

In a similar manner, the clonogenic assay showed lower colony formation ability of cisatracurium-treated HCT116 cells compared to unexposed HCT116 cells (Fig. 1C and D). The cisatracurium (10 μM or 20 μM) treated HCT116 cells had fewer cell colonies (166.7 ± 6.67 and 104 ± 4.73 respectively), while the untreated cells had mean colony number of 193.7 ± 3.48. Interestingly, 20 μM cisatracurium exposed cells did not form many compact colonies like what was observed in the unexposed cells and 10 μM cisatracurium exposed cells (Fig. 1C and D). Generally, colony-constituting cells of the exposed cells, especially for 20 μM cisatracurium, were loosely organized and exhibited some degree of loose physical contacts with neighboring cells of the same colony.

3.2. Cisatracurium induces G0/G1 arrest in HCT116 cells

Based on the observed retardation in HCT116 cells proliferation upon cisatracurium treatment, the effect of cisatracurium on cell cycle was investigated. Cisatracurium (10 μM or 20 μM) treated

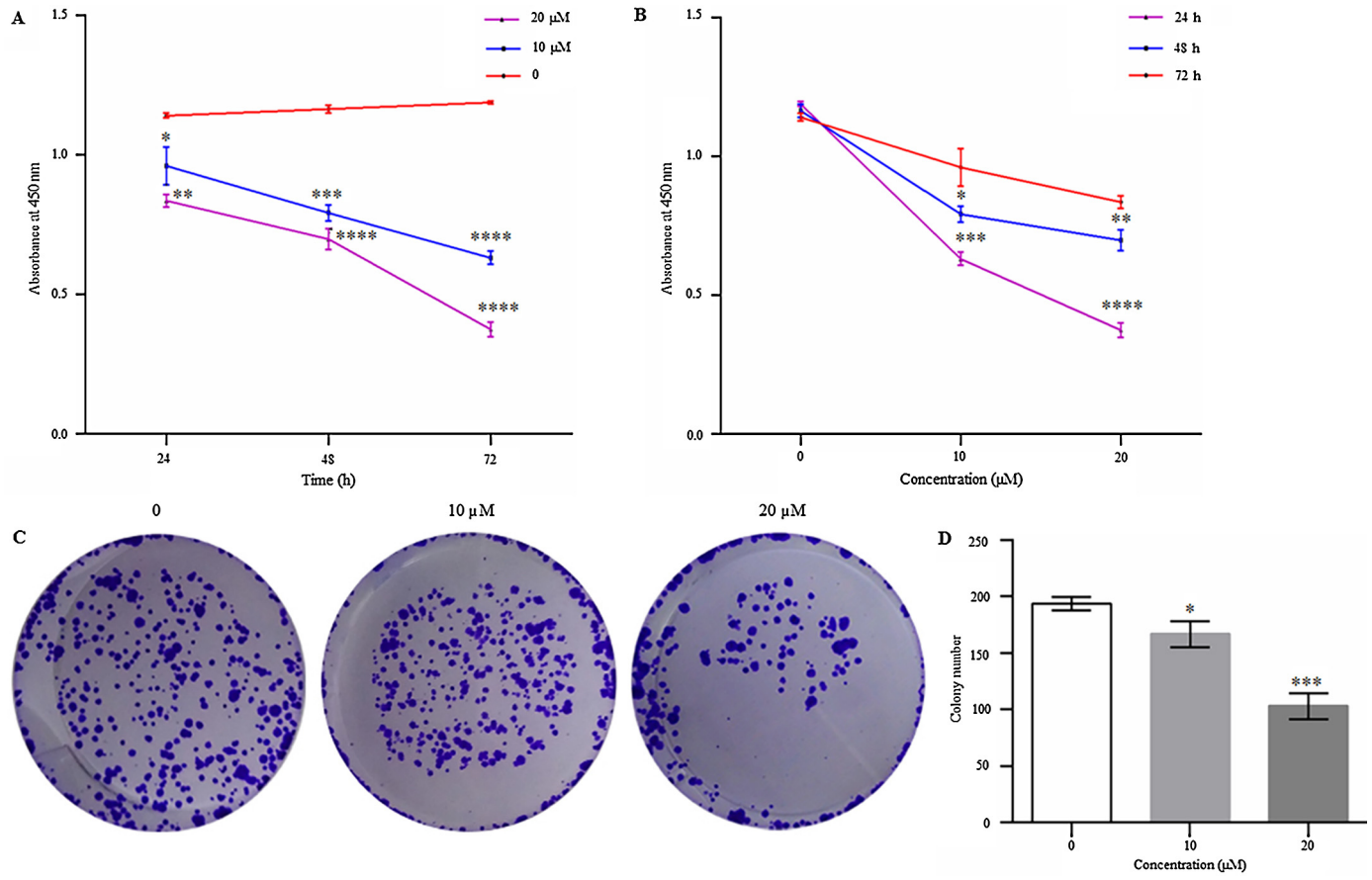

Fig. 1. Cisatracurium suppresses colon cancer (HTC116) colony formation. Following exposure to cisatracurium (10 μM or 20 μM), cells growth was assessed using CCK-8 assay and analyzed by MultiscanGo photospectrometer. (A) Time dependent assessment of proliferation of untreated colon cancer cells (0) and cisatracurium-treated cells. (B) Cisatracurium concentration dependent assessment of colon cancer cells proliferation. h in (A) and (B) represent hour. Data are presented as mean ± SEM ($n = 3$). Statistical significant differences in colon cancer cells growth suppression ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ versus control (0) and 24 h respectively) were observed. (C) HCT116 colonies formed in control (0) and treatment groups (10 μM and 20 μM). (D) Graphical presentation of number of colonies in untreated and treatment groups. Data are expressed as mean ± SEM ($n = 3$). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control.

HCT116 cells exhibited suppressed cell growth in G1 phase of cell cycle (Fig. 2A–C). An average of 46.84 ± 0.47%, 44.12 ± 0.92% and 9.04 ± 0.98% of the cell population were in G1, S and G2 phases, respectively, for untreated HCT116 cells, whereas 10 μM and 20 μM cisatracurium exposed HCT116 cells had 59.82 ± 0.90%, 28.78 ± 1.28% and 11.4 ± 1.30%, and 78.31 ± 1.69%, 14.09 ± 1.01% and 7.6 ± 0.90% of the cell population in G1, S and G2 phases, respectively (Table 2). Significant differences were observed in cells accumulation at G0/G1 and at S phase respectively ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control) (Table 2). Diminished cell cycle transition from G1 phase to S phase, upon cisatracurium treatment was concentration dependent.

Further molecular investigations including qRT-PCR and western blot indicated that cisatracurium exposure

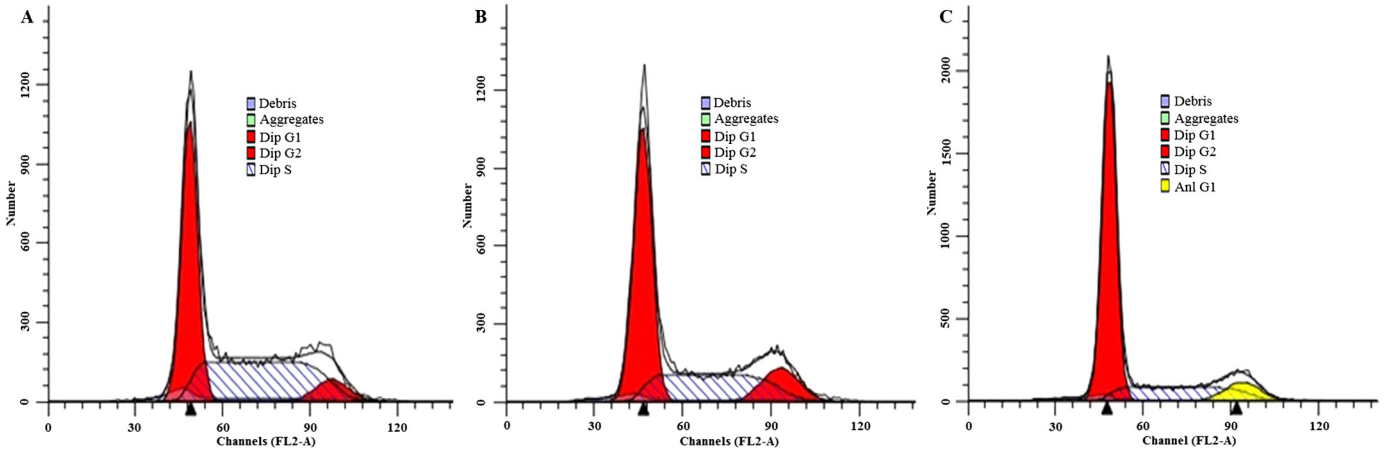

Fig. 2. Cisatracurium represses G0/G1-S cell cycle transition of HCT116 cells. (A) Flow cytometry analysis of untreated HCT116 cells (0). (B) Flow cytometry analysis of 10 μM cisatracurium treated HCT116 cells. (C) Flow cytometry analysis of 20 μM cisatracurium treated HCT116 cells.

Table 2
Cell cycle analysis of colon cancer cells (HCT116) after cisatracurium exposure.

Concentration (μM)	Cell cycle phases (%)		
	G0/G1	S	G2/M
0	46.84 ± 0.47	44.12 ± 0.92	9.04 ± 0.98
10	59.82 ± 0.90*	28.78 ± 1.28**	11.4 ± 1.30
20	78.31 ± 1.69***	14.09 ± 1.01***	7.6 ± 0.90

Data is percentage distribution of cells at cell cycle check points expressed as mean ± SEM ($n = 3$).

downregulated CD1, E2F, CE1 and p21 but upregulated p53 in a concentration dependent fashion (Fig. 3A–C). Cleavage of p21 was detected in cisatracurium-treated HCT116 cells. Mean fold change in CD1, E2F, CE1, p53 and p21 mRNA expression of untreated HCT116 cells was 1. The fold change in CD1, E2F, CE1, p21 and p53 mRNA expression of cisatracurium-treated cells (10 μM and 20 μM) normalized to β-actin and relative to the untreated HCT116 cells were 0.4 and 0.16 for CD1, 0.38 and 0.14 for E2F, 0.37 and 0.21 for CE1, 1.71 and 2.43 for p53, and 0.82 and 0.66 for p21. Significant differences in the level of mRNA and protein expression normalized to β-actin were observed (Fig. 3A and C).

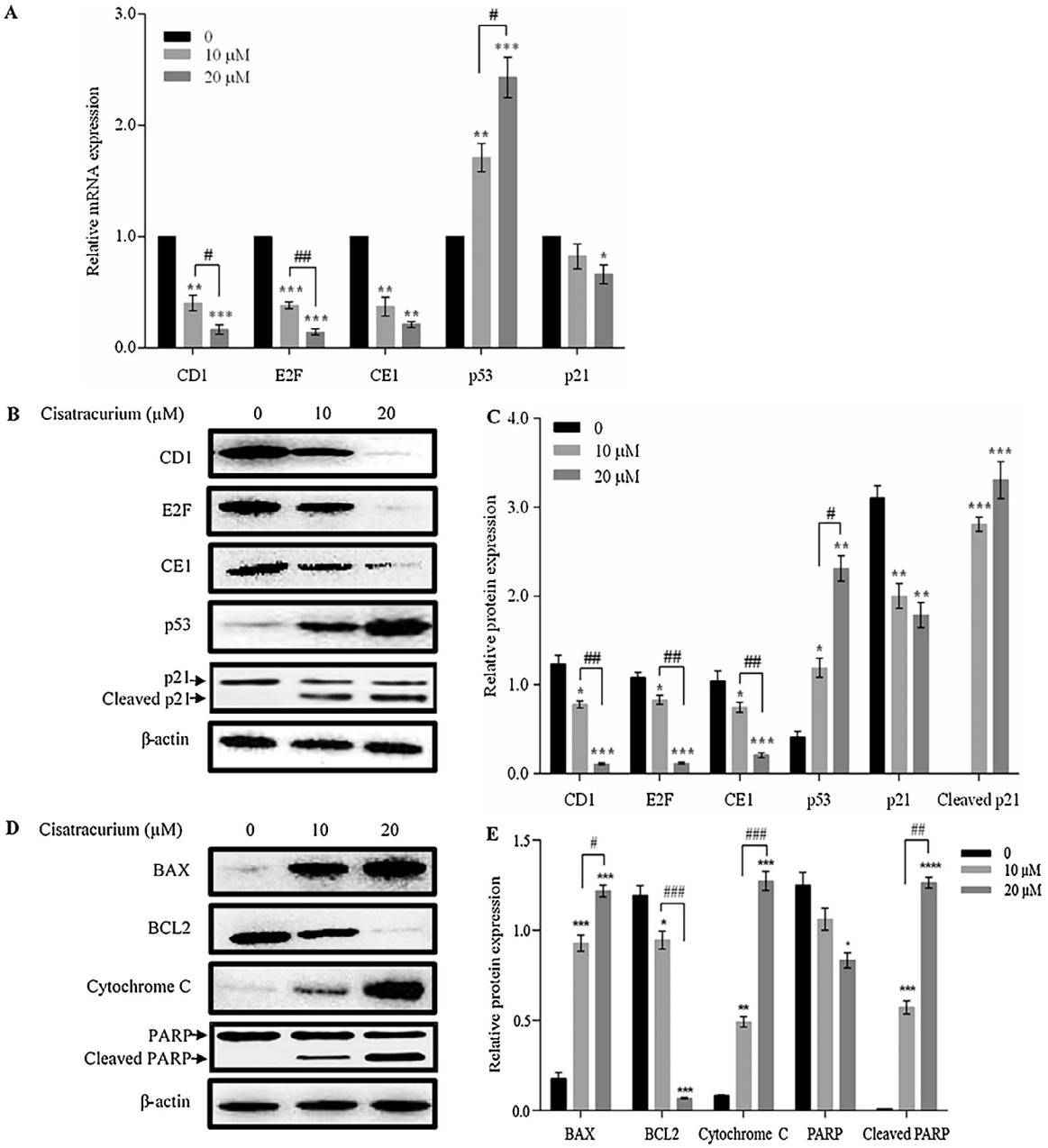

Fig. 3. Cisatracurium alters cell cycle regulatory genes, survival and intrinsic apoptotic regulatory proteins in HCT116 cells. (A) Cluster bar charts of CD1, E2F, CE1, p53 and p21 mRNA expression of untreated and cisatracurium-treated HCT116 cells. (B) CD1, E2F, CE1, p53, p21, cleaved p21 and β-actin proteins expression of untreated (0) and cisatracurium-treated groups (10 μM or 20 μM) following western experiment. (C) Cluster bar charts of CD1, E2F, CE1, p53, p21, cleaved p21 proteins expression normalized to β-actin. (D) BAX, BCL2, cytochrome C, PARP, cleaved PARP, and β-actin proteins expression of untreated (0) and cisatracurium treated groups (10 μM or 20 μM) after western blot experiment. (E) Cluster bar charts of BAX, BCL2, cytochrome C, PARP, cleaved PARP protein expression normalized to β-actin. β-actin was used as internal control during qRT-PCR and western blot experiments. Data are mean ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus untreated (0) $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ versus 10 μM.

3.3. Cisatracurium causes apoptosis of HCT116 cells

Using FITC-Annexin V and propidium iodide staining, it was evident that cisatracurium-treated HCT116 cells showed significant early onset of apoptosis compared to untreated cells (Fig. 4A). The total percentage of apoptotic cells (early stage and late stage) of cisatracurium-treated HCT116 cells was 19.99 ± 1.3 and 30.34 ± 1.16 compared to 9.49% for untreated HCT116 cells by 24 h (Fig. 4B). After 48 h of cisatracurium treatment, the percentage of total apoptotic cells for the untreated, 10 μM and 20 μM cisatracurium-treated HCT116 cells were 10.28 ± 1.27, 27.31 ± 0.33% and 47.12 ± 1.09%, respectively. The total percentage of apoptotic at 72 h for untreated HCT116 cells, 10 μM and 20 μM cisatracurium-treated HCT116 cells were 11.93 ± 1.92%, 42.59 ± 1.19 and 86.76 ± 2.68%. Significant differences in total percentage apoptosis were observed ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control) (Fig. 4B). Similar trends of significant difference were observed in total percentage apoptosis of HCT116

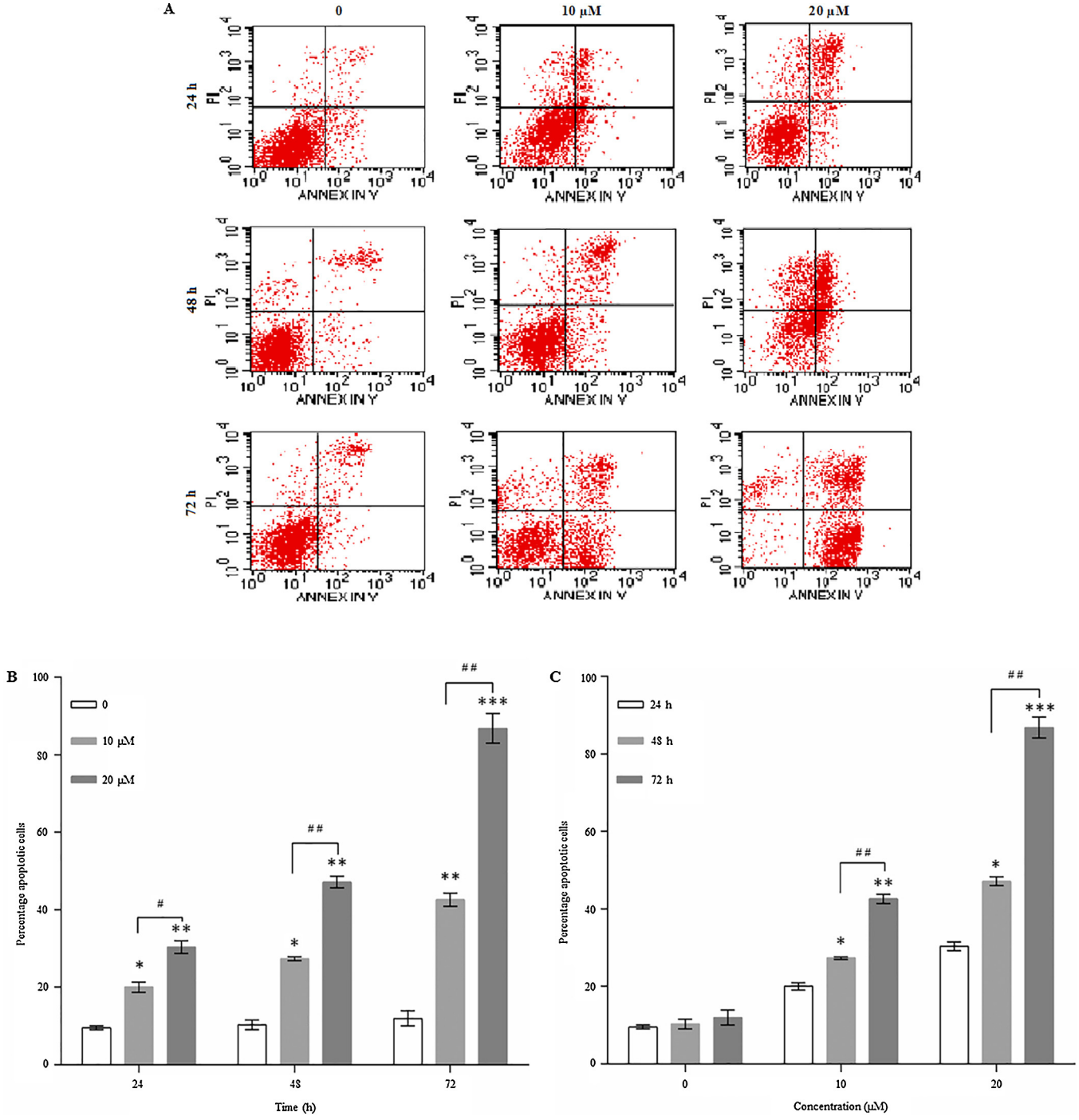

Fig. 4. Cisatracurium induces apoptosis in HCT116 cells. (A) FACS analysis output of untreated cells (0) and cisatracurium-treated cells (10 μM or 20 μM) at 24 h, 48 h and 72 h. (B) Cluster bar chart of percentage apoptotic cells of untreated (0) and cisatracurium-treated groups (10 μM and 20 μM) at various time points (24 h, 48 h and 72 h). (C) Cluster bar chart describing percentage apoptotic cells at 24 h, 48 h and 72 h in untreated (0) and cisatracurium-treated groups (10 μM and 20 μM). Data are expressed as mean ± SEM ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus untreated (0) and 24 h respectively. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus 10 μM, $^{\#\#}P < 0.01$ versus 48 h.

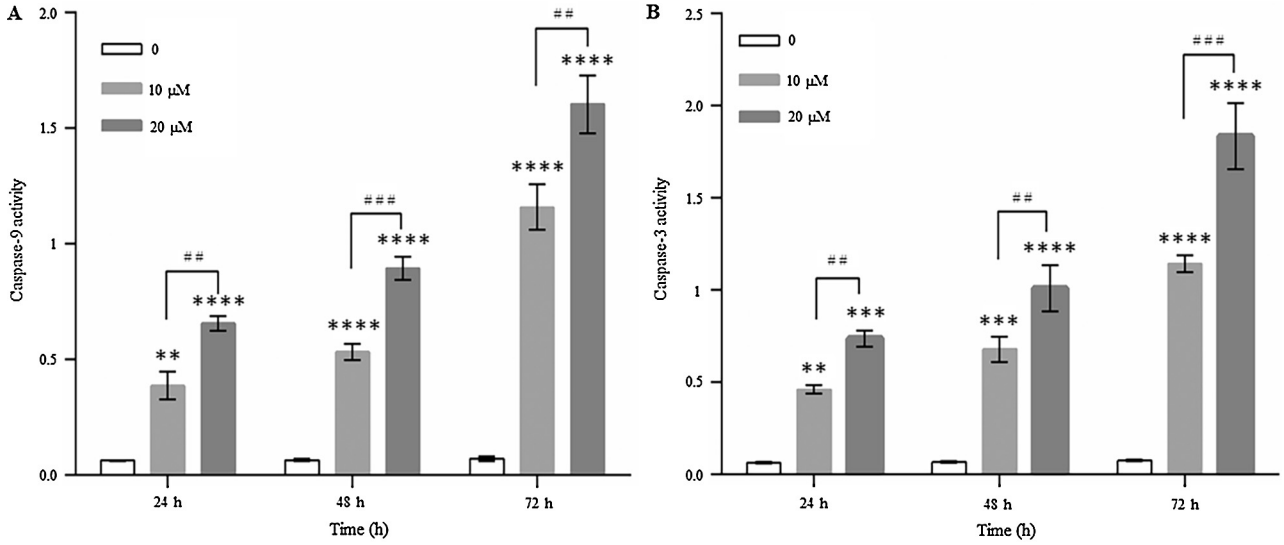

Fig. 5. Cisatracurium triggers caspase-9 and caspase-3 activities in HCT116 cells. (A) Bar chart of caspase-9 activity. (B) Bar chart of caspase-3 activity. Data are mean ± SEM ($n = 3$). $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ versus untreated group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ and $^{\#\#\#\#}P < 0.0001$ versus 10 μM.

cells at constant concentrations with varied time-points (Fig. 4C). Thus, the ability of cisatracurium to induce apoptosis of HCT116 cells was concentration and time dependent.

To investigate the molecular mechanism(s) underlying this observation, we determined some apoptotic proteins which could be implicated in cisatracurium-induced cell death. The expression levels of BAX, BCL-2, cytochrome C, PARP and its cleaved product were examined in both cisatracurium-treated and untreated HCT116 cells. Cisatracurium concentration-dependent upregulation of BAX and cytochrome C in HCT116 cells with concomitant downregulation of BCL-2 (Fig. 3D) compared with untreated HCT116 cells were observed. Cleaved PARP products were expressed in only the cisatracurium treated HCT116 cells (Fig. 3D). Significant differences in the level of protein expression (normalized optical density ratio of individual proteins to β-actin) were observed ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ versus control) (Fig. 3E). Caspase activity investigation indicated that caspase-9 and caspase-3 activities were significantly elevated in concentration-dependent manner, following exposure of HCT116 to cisatracurium (Fig. 5) ($^{***}P < 0.001$ $^{****}P < 0.0001$ versus control) (Fig. 5A and B). Overall, the results suggested that apoptosis was mediated via p53-dependent intrinsic apoptotic pathways.

3.4. Cisatracurium enhances 5-fluorouracil (5-FU) sensitivity in HCT116 cells

The effect of cisatracurium (10 μM and 20 μM) on 5-FU (10 μM) efficacy was investigated in HCT116 cells. Following treatment of HCT116 cells with 5-FU alone and 5-FU with cisatracurium, the efficacy of 5-FU dramatically improved as evidenced by results of the CCK-8 cytotoxicity assay, where 5-FU alone and cisatracurium-5-FU treated cells growth decreased remarkably (Fig. 6A and B). In comparison with untreated HCT116 at 24 h, growth of HCT116 cells was inhibited by 28%, 43%, and 66% for 5-FU, 5-FU/10 μM cisatracurium and 5-FU/20 μM cisatracurium, respectively. At 48 h, the rate of HCT116 cells growth inhibition increased to 35%, 61% and 86% for 5-FU, 5-FU/10 μM cisatracurium and 5-FU/20 μM

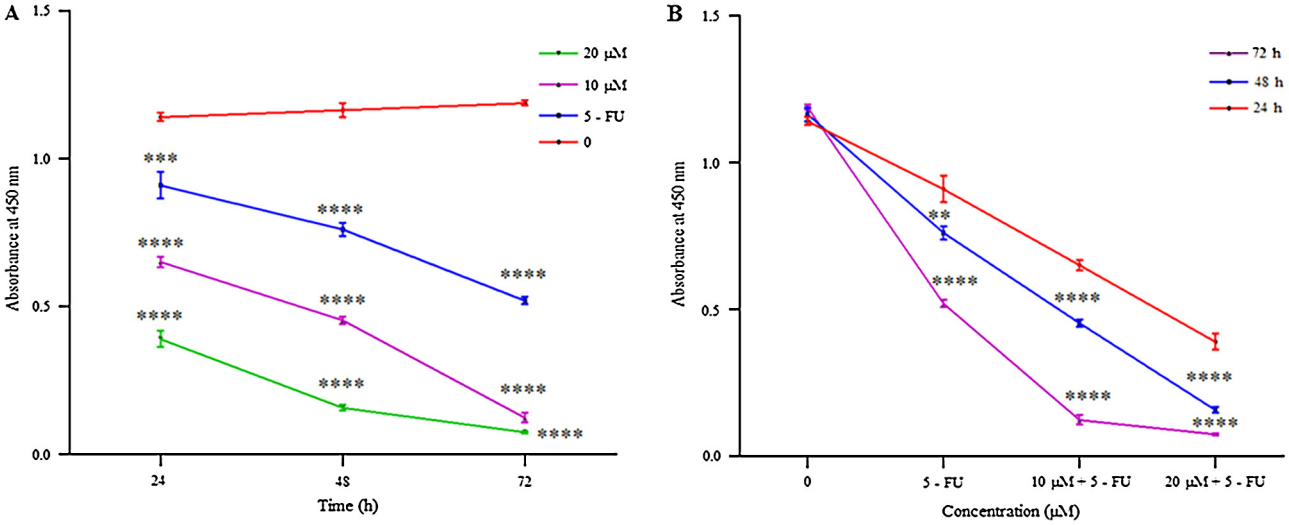

Fig. 6. Concurrent exposure of HCT116 cells to cisatracurium and 5-FU enhances the sensitivity of 5-FU. (A, B) HCT116 cells inhibition in 5-FU (10 μM) alone or 5-FU (10 μM) with various concentrations of cisatracurium (10 μM and 20 μM) and at various time points (24 h, 48 h, 72 h) respectively. Data are expressed as mean ± SEM ($n = 3$). $^{***}P < 0.001$ and $^{****}P < 0.0001$ versus control (0). $^{**}P < 0.01$ and $^{****}P < 0.0001$ versus 24 h.

cisatracurium, respectively as compared with untreated HCT116 cells. An overwhelming growth repression was observed at 72 h; 56%, 89% and 94% for 5-FU, 5FU/10 μM and 5-FU/20 μM cisatracurium, respectively compared with control. However, when HCT116 cells growth in each treatment concentration was analyzed and compared on time bases, we observed that in comparison with 24 h proliferation in the untreated cells increased by 2% and 4% for 48 h and 72 h respectively. On the contrary, in comparison with 24 h, HCT116 cells proliferation 5-FU was inhibited by 16% and 43% for 48 h and 72 h respectively. In the 5-FU/10 μM, proliferation was decreased by 30% and 81% for 48 h and 72 h respectively compared with 24 h. In comparison with 24 h, 5-FU/20 μM further increased HCT116 inhibition by 57% and 83% for 48 h and 72 h respectively. Figures A and B demonstrate concentration and time-dependent inhibition of HCT116 cells growth. Significant differences in growth inhibition were observed ($^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ versus control and 24 h).

4. Discussion

Cells function according to well-structured rules that govern their growth and death. These rules are partly enshrined in the cell cycle check points. However, these rules can be altered through genetic mutations to give rise to uncontrollable dividing cells, termed cancer cells.

The turnover of mammalian cells is controlled by a sequence of positive and negative regulatory factors in the cell cycle. The main molecular activity leading to cancer development is disruption of cell cycle checkpoints often mediated by dysregulation of cell cycle regulatory proteins [21,22]. The D type of cyclins, is among the regulatory proteins of cell cycle. It is produced in response to stimulation of growth factors. Transition of the cell cycle from G1 phase to the DNA synthesis phase (S) is regulated by accumulation of the D family of cyclins (D1, D2, and D3) with their associated kinases (CDKs) [23]. Upregulation of cyclin D can exacerbate progression through the G1 phase. Cyclin D1 is capable of regulating the activities of CDK4. Cyclin D1-CDK4 complex phosphorylates retinoblastoma protein (pRb), the product of the retinoblastoma gene (a well-known tumor suppressor). Inactivation of pRb liberates the E2F family of proteins which play essential function in cell cycle progression by inducing the expression of genes necessary for entry into S phase. These include S phase regulatory factors CDK2, cyclin E and cyclin A [24]. E2F family members E2F-1, E2F-2, and E2F-3 binds to pRb, and the complexes E2F-pRb formed are basically located in G1 [25,26]. Based on the above cell cycle regulatory machinery, inhibition of cyclin D can repress cyclin E expression by inhibiting Rb phosphorylation and E2F expression to prevent progression to S phase in the cell cycle. Expression of cyclin D, E2F and cyclin E were suppressed in HCT116 cells following treatment with cisatracurium. This may explain the observed concentration-dependent inhibition of HCT116 cell proliferation, colony formation and G1 phase arrest in the cell cycle assessment of cisatracurium-treated HCT116 cells compared to untreated HCT116 cells.

p21 is a member of the Cip/Kip family of the cyclin-dependent kinases (CDK) inhibitor proteins. It is a potent inhibitor of cell cycle progression and partly regulated by p53 [27–29]. Cisatracurium treatment caused cleavage and concentration-dependent inhibition of p21 expression. Conversely, cisatracurium treatment induces increased p53 expression in HCT116 cells. This suggests that the arrest at G1 phase of the cell cycle is p21 independent. The repression of p21 could be due to the increased activity of Caspase-3 in the cisatracurium-treated cells that underwent apoptosis. In the light of these findings, it is believed that cisatracurium might be causing continuous DNA damage in the human colorectal carcinoma cells.

Cell death includes necrosis and cell suicide (apoptosis). Apoptosis is programmed cell death that prevent parent cells with damaged DNA from transferring the unrepaired DNA, which is capable of causing genetic mutation, to their daughter cells. Many malignancies, including human colorectal carcinoma, are characterized by compromised genomic integrity as a result of unrepaired DNA damages which lead to genetic mutations. In an attempt to cure this mischief, physiological cellular p53 degradation is aborted to allow p53 accumulation to further stimulate a cascade of cellular events which will enable repair of the damaged DNA. However, if DNA repair fails, p53 accumulation increases and stimulates the process of cell death in order to eliminate the damaged cells [27,30,31]. Also, Polyak et al. reported that overexpression of p53 induces apoptosis only when p21 fails to increase proportionally, and conversely, overexpression of p21 blocks p53-induced apoptosis [32]. The observed increased level of p53, but decreased level of p21 expression and released of its cleaved products in HCT116 cells suggest that cisatracurium could be causing DNA damage but the impaired repair of the damage promoted p53 accumulation to eventually induce apoptosis of the cisatracurium-treated cells (Fig. 4A–C).

To gain more insight into the mechanism that underlies the above observation, the expression of key proteins that are important players in apoptosis were assessed (Fig. 4). Cytochrome C and BAX exhibited concentration-dependent overexpression in response to cisatracurium treatment of HCT116 cells while the expression of BCL-2, decreased as compared with untreated HCT116 cells (Fig. 4D and E). Also, Caspase-9 and Caspase-3 activities were increased in cells treated with cisatracurium (Fig. 5A and B). BAX is a member of the pro-apoptotic family of proteins which induce the opening of mitochondrial voltage-dependent anion to release Cytochrome-C and other pro-apoptotic factors from the mitochondria. These occurrences result in activation of caspases [33,34]. They function as principal proteins of apoptosis to ensure that cells harboring unrepaired damages are quickly destroyed and eliminated. The increased expression of BAX and decreased expression of BCL-2 in cisatracurium-treated cells confirmed the type of cell death observed as apoptosis. Caspase-3 is a major executioner protein (effector caspase) of apoptosis which is activated in cells undergoing apoptosis by both extrinsic (death ligand) and intrinsic (mitochondrial) pathways. It is present in cells as a zymogen and remains dormant until it is cleaved by initiator caspases such as Caspases-8 and 9 [35,36]. It has been reported that increase Caspase-3 activity cleaves p21 and promote apoptosis [37,38].

To confirm these observations, caspase activity and PARP cleavage, which are known indicators of apoptosis were assessed [39–41]. Both Caspase-9 and Caspae-3 activities were increased in the cisatracurium treated cells compared to control. Cleaved PARP was also detected in the cisatracurium treated cells. Consistent with these findings, the FITC Annexin V/propidium iodide staining revealed marked percentage of apoptotic cells in cisatracurium treatment concentrations. Thus, cisatracurium could effectively induced apoptosis in HCT116 cells through at least, the intrinsic apoptotic pathway.

Continuous infusion of cisatracurium in patients undergoing surgery usually ranges from 2 to 8 h depending on the type of surgery [42–45]. However, continuous intravenous infusion for prolong period (48–72 h) of cisatracurium may be required in the event of patient-ventilator asynchrony and in patient with imminent respiratory failure requiring ventilation support. Inferring from the inhibitory effect of cisatracurium on HCT116 cells demonstrated herein, the use of cisatracurium for muscle relaxation in surgeries for tumor excision, especially in colorectal cancer resections may impact positively on the surgical treatment. However, anesthesiologists and intensivists need to be vigilant, as

excessive and prolong use, especially in the critical care settings could be fatal to noncancerous cells in patients.

5-FU is heterocyclic aromatic organic compound with a chemical architecture similar to DNA and RNA pyrimidine molecules. It is an analog of uracil with a fluorine atom at the C-5 position in place of hydrogen [46]. 5-FU is a chemotherapeutic drug that disrupts nucleotide synthesis through RNA misincorporation [47]. The rapid catabolism of 5-FU by dihydropyrimidine dehydrogenase (DPD) enzyme limits the therapeutic efficacy of the drug. 5-FU is extensively used for colorectal cancer treatment. The enhanced inhibitory effect of combined 5-FU and cisatracurium suggests a synergistic therapeutic effect which could be due to at least enhanced apoptosis of HCT116 cells.

5. Conclusion

This study provides insides into the effects of cisatracurium on the behavior of human colorectal cancer cells and the related molecular mechanisms. Cisatracurium could effectively inhibit the proliferation of HCT116, a colon cancer cell line, and induce apoptosis via the intrinsic apoptotic pathway in a concentration and time dependent manner. Also, cisatracurium and 5-FU synergistically inhibited the proliferation of HCT116 cell in vitro.

Conflict of interest

The authors declare no conflicts of interest concerning this article.

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