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Sequential administration of camptothecin sensitizes human colon cancer HCT116 cells to paclitaxel via $p21^{Cip1/WAF1}$

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Colorectal cancer is the third leading cause of cancer-related death in Western countries. Chemotherapeutic agents with different mechanisms of action have shown an increase in cure rates. In the present study, we investigated the effect of a combination of low concentration of paclitaxel (taxol, 5 nM) and topoisomerase I inhibitor camptothecin (CPT) on HCT116 colon cancer cells. Although the viability of cells treated with taxol alone was similar to that of control cells, sequential treatment with taxol and CPT exhibited high cytotoxicity. However, the opposite sequence of treatment did not exert cytotoxic effects on HCT116 cells. This enhanced cytotoxicity of the sequential combination therapy was the result of mitotic arrest, which increased the level of $p21^{Cip1/WAF1}$ through the p38 mitogen-activated protein kinase (MAPK) pathway. Knockdown by $p21^{Cip1/WAF1}$ siRNA or treatment with a p38 inhibitor reduced the viability of cells sequentially exposed to taxol and CPT. Taken together, a low taxol concentration in combination with CPT induced mitotic arrest in HCT116 cells, leading to synergistic cell death through enhanced expression of $p21^{Cip1/WAF1}$ and p38 MAPK pathway. Therefore, taxol could play a role as a sensitizer of CPT in colon cancer cells.

Keywords: topoisomerase I; taxol; camptothecin; colon cancer; p21; HCT 116 cells

Introduction

Colorectal cancer is the third leading cause of cancer-related death in Western countries. (Segal and Saltz 2009). Ongoing clinical trials are evaluating how best to combine the currently available therapies in order to prolong patient life and increase cure rates (Wolpin and Mayer 2008). Chemotherapeutic agents with different mechanisms of action have exhibited high response rates (Fishman and Wadler 2001; Abal et al. 2004). Camptothecin (CPT), found in the *Camptotheca acuminate* plant native to China, is a topoisomerase I inhibitor (Wall et al. 1966; Kaufmann et al. 1996). CPT interferes with DNA replication. The collision between topoisomerase I cleavage complexes and DNA replication forks generates double-strand breaks during DNA replication (Pommier 2006). The combination of irinotecan, a CPT analog, and 5-fluorouracil/leucovorin has been approved as a first-line chemotherapy for colorectal cancer patients (Fuchs et al. 2006; Bathe et al. 2009). However, the high concentration of CPT exerts adverse effects such as diarrhea and neutropenia. In cells containing wild-type $p53/p21^{Cip1/WAF1}$, the low CPT concentration does not induce cell death but cell cycle arrest, and prolonged CPT treatment eventually leads to senescence (Gupta et al. 1997; Zhou et al. 2002).

Paclitaxel (taxol) is an anti-mitotic drug that affects microtubule assembly, thereby disrupting microtubule bundle formation (Manfredi et al. 1982). Currently, taxol is approved as a first-line chemotherapy drug for breast and ovary cancers. Taxol has different cellular effects depending on its concentration. In human colon cancer HCT116 cells, a high concentration of taxol (>100 nM) induces cell death, whereas a low dose of taxol (<10 nM) results in mitotic delay, producing multinucleated cells upon prolonged exposure (Torres and Horwitz 1998; Giannakakou et al. 2001).

Although combination chemotherapy with taxol and CPT drugs has been administered for breast, cervical, and lung cancers for a decade, the effects of this therapy are still controversial. Furthermore, its clinical effects on colorectal cancer are currently unknown. Therefore, in the present study, we evaluated the cytotoxic effect of sequential low taxol concentration and CPT on human colon cancer HCT116 cells.

Materials and methods

Cell culture and reagents

Human HCT116 colon carcinoma cells were grown in complete growth media containing Dubelco's modified Eagle medium supplemented with 10% fetal bovine serum and incubated in a humidified incubator at 37°C with 5% CO_2. HCT116 cells were treated with taxol (T7402; Sigma, MO, USA) and CPT (C9911; Sigma, MO, USA). SP600125 (420119; Calbiochem,

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Darmstadt, Germany), SB203580 (559389; Calbiochem, Darmstadt, Germany), and nocodazole (332 μM; 487928; Calbiochem, Darmstadt, Germany) were prepared in dimethyl sulfoxide. Cycloheximide (239763; Calbiochem, Darmstadt, Germany) was dissolved in ethanol. Cells were treated with taxol, CPT, or a combination as indicated in Results under strictly identical conditions. For the p38 inhibitor study, cells were exposed to SB203580 before taxol treatment. For mitotic cell synchronization, nocodazole-arrested mitotic cells were obtained by the mechanical shake-off procedure. Briefly, cells were treated with nocodazole (200 nM) for 15 h, and then were detached by the mechanical shaking procedure to collect mitotic cells. Mitotic cells were cultured with complete growth media containing CPT drug for the indicated time.

Small interference RNA transfection

In preparation for transfection, cells were plated in 60-mm plates and incubated overnight. Cells were transfected with their respective small interference RNAs (siRNAs) using HiPerFect reagent according to the manufacturer's protocol (Qiagen, MD, USA). The sense and antisense target sequences of the p21 siRNA were 5′-UGGCGGGCUGCAUCCAGGA-3′ and 5′-UCCUGGAUGCAGCCCGCCA-3′, respectively. The control siRNA sequences were 5′-CGU ACGCGGAAUACUUCGA-3′ (sense) and 5′-UCGA AGUAUUCCGCGUACG-3′ (antisense). siRNAs used were synthesized by Bioneer Inc. (Daejeon, Korea). After transfection, cells were cultured for 6 h. The transfection media was then removed and replaced with complete growth media. Cells were grown for an additional 24 h period and treated with drugs. Knockdown of target proteins was confirmed by Western blot.

Trypan blue exclusion assay

HCT116 cells were plated at 6×10^4 in a 24-well plate. Cells were treated with either taxol, CPT, or their sequential combination at the indicated time points. Upon completion of incubation, viability was assessed after adding trypan blue solution (4% in PBS) to the culture medium.

Cell cycle analysis by flow cytometry

HCT116 cells were harvested in ice-cold PBS and fixed with 70% ethanol. They were then washed with PBS and treated with 100 μg/ml RNase (R6513; Sigma, MO, USA) and stained with 0.1 mg/ml propidium iodide (PI) (P4170; Sigma, MO, USA). Samples were analyzed using a FACS Calibur instrument (Becton Dickinson, NJ, USA). The percentage of cells in the subG1, G1, S and G2/M stages was determined from DNA content histograms by CELLQuest for Windows (Becton Dickinson, NJ, USA).

Western blot analysis

Cell extracts were prepared with RIPA buffer (150 mM NaCl, 1% NP40, 0.1% sodium dodecyl sulfate (SDS), 2 mM EDTA, 6 mM Na_2HPO_4, 4 mM NaH_2PO_4, 50 mM NaF, 200 μM Na_3VO_4, and 1 × protease inhibitor). Protein concentrations were normalized using the Bradford assay (Bio-Rad Laboratories, CA, USA), and 20 μg of protein were separated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to nitrocellulose membrane. The membranes were incubated with antibodies against PARP (556494; BD Biosciences, NJ, USA), $p21^{Cip1/WAF1}$ (#05-345; Upstate, MA, USA), p53 (sc-126; Santa Cruz Biotechnology, CA, USA) or actin (A2066; Sigma, MO, USA). Primary antibodies were detected using horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) and an enhanced chemiluminescence kit (Santa Cruz Biotechnology, CA, USA).

Mitotic index

Cells were fixed with 4% (w/v) paraformaldehyde-PBS, permeabilized with 0.1% (v/v) NP-40, and stained with 0.1 mg/ml 4′,6-diamidino-2-phenylindole (DAPI) (Sigma). For each sample, at least 1000 cells were randomly counted by fluorescence microscopy, and mitotic cells were scored by their lack of nuclear membrane and evidence of chromosome condensation. Mitotic index was calculated as the number of mitotic cells per total number of cells.

Apoptosis assay

The ability of taxol or CPT to induce apoptosis of HCT116 cells was measured by an annexin V-fluorescein isothiocyanate (FITC) apoptosis kit according to the manufacturer's protocol (550911; BD, NJ, USA).

Statistical analysis

All data are expressed as mean ± SEM obtained from three independent experiments. Data were analyzed by the analysis of variance (ANOVA) test. $P < 0.01$ was used to indicate statistical significance.

Results

The effect of low concentrations of taxol and CPT on cell viability of HCT116 cells

Treatment with a low concentration of taxol or CPT arrests the cells at a specific cell cycle phase instead of undergoing cell death; therefore, we first investigated the effect of low taxol concentration and CPT on the viability of colon cancer HCT116 cells. Based on previous studies, we set the concentrations of taxol and CPT at 5 and 20 nM, respectively, to minimize cellular toxicity (Torres and Horwitz 1998; Zhou et al. 2002). After treating the HCT116 cells with 5 nM taxol or 20 nM CPT, cell viability was measured in a time-dependent manner. As shown in Figure 1A, cells exposed to taxol for 12 h were not different from control cells, and even at 36 h post-treatment the cell population decreased by only 10%. CPT treatment also showed no differences in cell viability at 24 h as compared to the control group. At 36 h after CPT addition, cell viability reduced by 20%. Cells treated with both drugs simultaneously were similar to cells treated with CPT alone. We determined the IC_{50} for taxol and CPT to be 65 and 100 nM, respectively. These results suggest that the low concentration of taxol and CPT did not exert serious cytotoxic effects. Next, we determined the cell cycle profile of HCT116 cells in response to taxol or/and CPT. At 24 h after taxol treatment alone, 50% of the cell population was arrested at the G2/M phase (Figure 1B). After treatment with CPT alone or in combination with taxol, 40% of cells were arrested at the G1 phase. The population of S phase cells did not change regardless of drug treatment. We obtained similar results at 12 h post-treatment (data not shown). Taken together, these results indicate that the low concentrations of taxol or/and CPT were not cytotoxic to HCT116 cells.

Synergistic effects of sequential taxol and CPT treatment in human colon cancer cells

Since a low concentration of taxol or/and CPT was not cytotoxic to HCT116 cells, we investigated the effect of sequential treatment with taxol and CPT on HCT116 cells. We applied two different regimens for this

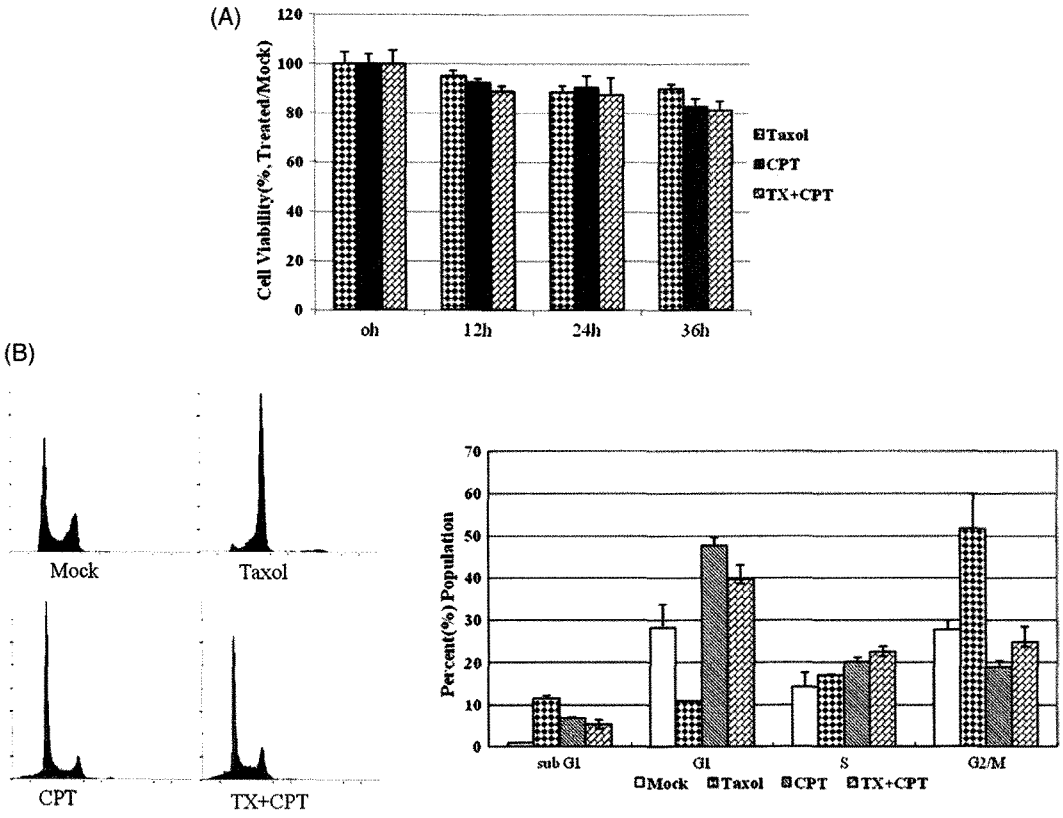

Figure 1. Cell viability following low-dose taxol and CPT treatment in HCT116 cells. (A) HCT116 cells were treated with taxol (5 nM) alone, CPT (20 nM) alone, or simultaneous combination of both drugs (TX + CPT) for the indicated time points. Cell viability was determined by the trypan blue exclusion assay. Data represent the average of three independent experiments. Error bars represent standard error. (B) Cellular DNA content was analyzed by flow cytometry. The proportion of cells in the G1, S, and G2/M phases are shown as histograms.

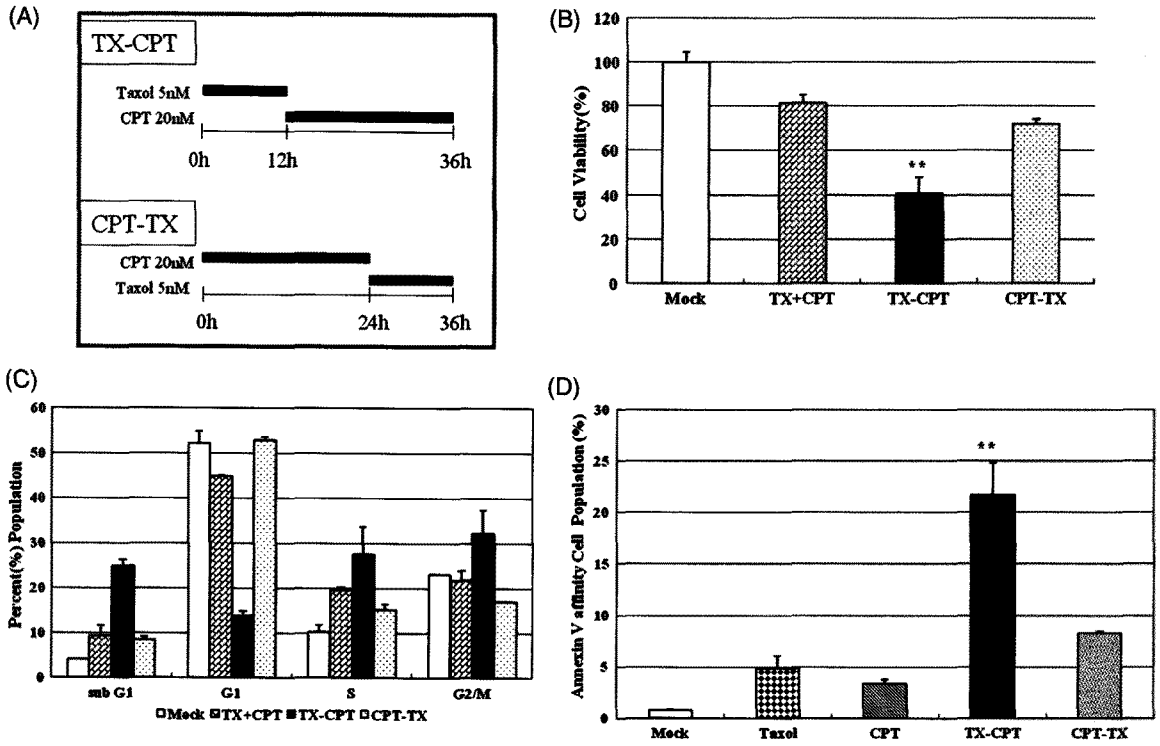

Figure 2. Schedule-dependent combination between taxol and CPT in HCT116 cells. (A) Protocol for the treatment of cells with combined taxol and CPT. HCT116 cells were exposed to taxol and CPT at various schedules; sequential exposure to taxol (5 nM) for 12 h followed by CPT (20 nM) for 24 h [TX-CPT] and sequential exposure to CPT (20 nM) for 24 h followed by taxol (5 nM) for 12 h [CPT-TX]. (B) Cells were treated with TX + CPT, TX-CPT, or CPT-TX. Cell viability was assessed by trypan blue exclusion assay. Control data for the drug treatments were obtained after incubation with DMSO (Mock). ** $P < 0.01$ vs. CPT-TX. (C) Cell cycle analysis for HCT116 cells by flow cytometry. Cells were treated for 36 h with different schedules, and cell cycle analysis was evaluated by PI staining. Cell cycle distribution of three independent experiments is shown as the subG1 (typical of apoptotic cell death), G1, S and G2/M phases. (D) Apoptotic cells induced by different schedules were observed by annexin V-FITC staining under fluorescence microscopy. Data represent the average of three independent experiments. Error bars represent standard error. ** $P < 0.01$ vs. mock.

experiment (Figure 2A). The cells were exposed sequentially to taxol (5 nM) for the first 12 h and then to CPT (20 nM) for 24 h (TX-CPT regimen), or vice versa (CPT-TX regimen). Cell death was not induced in TX + CPT-exposed cells; however, sequential drug treatments showed different results. In cells exposed to taxol and CPT simultaneously (TX + CPT regimen) for 36 h, the viability decreased by 20%, which is similar to the data shown in Figure 1A, whereas the viability of cells treated with the TX-CPT regimen decreased by 60% (Figure 2B). In the CPT-TX-exposed cells, the cell population was reduced by 30%. These data suggested that the TX-CPT regimen exerted a synergistic effect on HCT116 cells as compared to other regimens. Using flow cytometry, we determined the cell cycle profile of cells exposed to either the TX-CPT or the CPT-TX regimen. As expected, the TX + CPT- and CPT-TX-exposed cells were arrested at G1, similar to the control group (Figure 2C). However, the number of TX-CPT-exposed cells arrested at the G2/M phase was more than the cells arrested at G1 phase. After the TX-CPT regimen, the subG1 cell population also increased by 25%. To verify cell death induced by the TX-CPT regimen, we conducted an annexin V staining assay. Among the cells exposed to taxol (5 nM) alone for 12 h or CPT (20 nM) alone for 24 h, annexin V positive cells were 5% and 3%, respectively. Further, 8% of CPT-TX-exposed cells were annexin V positive, whereas in the TX-CPT regimen group, the number of apoptotic cells increased by 22% (Figure 2D). These results show that the TX-CPT regimen induces cell death by a synergistic interaction in HCT116 cells.

Cytotoxic effect of taxol combined with CPT in HCT116 cells via mitotic arrest

In order to determine whether the G2/M phase-arrested cells treated by the TX-CPT regimen are in the G2 or M phase, we determined the mitotic index.

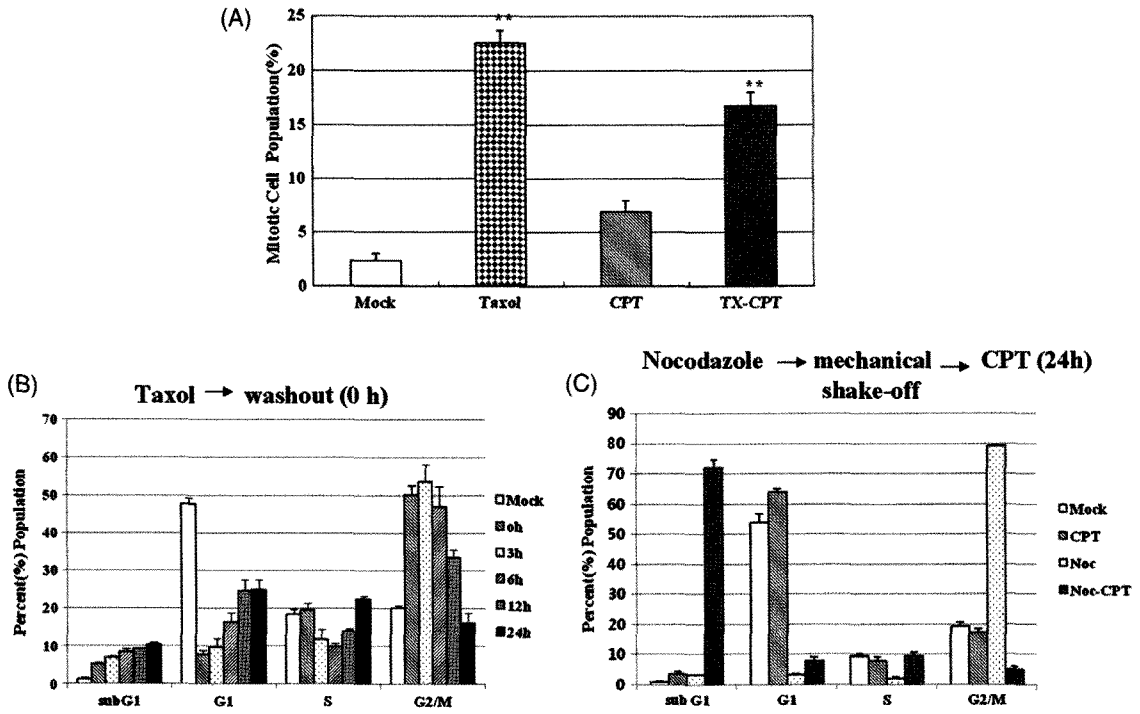

Figure 3. Mitotic arrest in cells exposed to the TX-CPT regimen. (A) HCT116 cells were incubated according to the indicated drug schedule. DAPI staining for mitotic cells was performed as described in Materials and methods. The mean ± SEM for three independent experiments is shown. ** $P < 0.01$ vs. mock. (B) After treatment with a low concentration of taxol (5 nM) for 12 h, cells arrested at mitosis were released into fresh drug-free media (0 h), and were sequentially incubated for the indicated time points. The cellular DNA content of 3 independent experiments was analyzed by flow cytometry. (C) HCT116 cells were treated with nocodazole (200 nM) for 15 h. Synchronized cells, isolated by mechanical shake-off procedure, were incubated with CPT (20 nM) for 24 h. Cell cycle analysis was evaluated by PI staining. The percentages of cells in subG1, G1, S and G2/M phases are indicated in the histogram.

As shown in Figure 3A, the TX-CPT treatment increased mitotic cells by 16%, whereas the CPT-TX regimen resulted in a 6% increase. Treatment with taxol (5 nM) alone also showed an increase of 20% in mitotic cells. These results suggest that the TX-CPT regimen induces mitotic arrest. Since taxol was removed from the medium before the cells were exposed to CPT in the TX-CPT regimen, in our next experiment we determined how long the taxol-induced mitotic arrest was maintained after removing taxol from the medium. HCT116 cells treated with taxol for 12 h were washed and then incubated in a drug-free complete medium for the indicated time points. After removing taxol, the number of cells in G1 phase gradually increased; however, 30% of the cells were still in mitosis (Figure 3B), suggesting that the addition of CPT after taxol treatment could affect taxol-dependent cytotoxicity toward mitotic cells. In addition, we investigated whether mitotic arrest is critical for CPT action. For this purpose, we used the spindle poison agent nocodazole, which plays a role in microtubule depolymerization during mitosis. After arresting cells at metaphase using nocodazole, cells were treated with CPT and the cell cycle profile was observed. Interestingly, 70% of cells pre-exposed to nocodazole underwent cell death in the presence of CPT (Figure 3C), indicating that CPT induces critical cytotoxicity to mitotic cells. In the present study, nocodazole had a greater cytotoxic effect than taxol, since CPT was introduced to nocodazole-synchronized mitotic cells through the mechanical shake-off procedure, but only 50% of taxol-treated asynchronized cells arrested at mitosis without mechanical shake-off. These results suggest that cells sequentially exposed to taxol and CPT exhibited enhanced cytotoxicity through synergistic drug interactions.

The induction of apoptosis through $p21^{Cip1/WAF1}$ protein and the p38 MAPK pathway in TX-CPT-exposed HCT116 cells

In order to determine the molecular mechanism of sequential taxol-CPT treatment in HCT116 cells, the expression of tumor suppressor gene p53 was analyzed by Western blot. Firstly, we investigated the expression of the apoptosis marker protein poly(ADP-ribose)

polymerase (PARP) to confirm the induction of cell death by the TX-CPT treatment. The pattern of PARP cleavage was not observed in cells exposed to taxol alone and was very faint in the CPT alone treated cells. However, PARP cleavage was clearly apparent in cells treated with TX-CPT (Figure 4A). The level of p53 protein in taxol-treated cells was similar to that in control cells, whereas its expression greatly increased in CPT-treated cells. Interestingly, $p21^{Cip1/WAF1}$ expression was enhanced in both cells exposed to taxol alone and CPT alone. Thus, taxol induced p53-independent expression of $p21^{Cip1/WAF1}$ and CPT enhanced the level of $p21^{Cip1/WAF1}$ in a p53-dependent manner. The expression of these proteins continued in the TX-CPT regimen cells (Figure 4A). In particular, the $p21^{Cip1/WAF1}$ protein was highly expressed in TX-CPT-treated cells, suggesting the synergistic effect of $p21^{Cip1/WAF1}$ expression. However, the level of p53 in TX-CPT-treated cells was similar to that of CPT-treated cells. To determine the mechanism of increased $p21^{Cip1/WAF1}$ expression, the TX-CPT-treated cells were treated with cycloheximide (a protein synthesis inhibitor) or MG-132 (a proteasome inhibitor). As shown in Figure 4B, cycloheximide-treated TX-CPT cells failed to synthesize $p21^{Cip1/WAF1}$, whereas the level of $p21^{Cip1/WAF1}$ and p53 was not altered in the presence of MG-132, indicating that the increase of $p21^{Cip1/WAF1}$ protein following TX-CPT treatment was induced by the activation of protein synthesis. Next, we determined whether the $p21^{Cip1/WAF1}$ protein is required for the induction of cell death in TX-CPT-treated cells using siRNA against $p21^{Cip1/WAF1}$. The expression of $p21^{Cip1/WAF1}$ was transiently knocked-down by an siRNA oligomer (Figure 5A). The cell viability of the TX-CPT regimen group decreased 60%, whereas the cell viability in $p21^{Cip1/WAF1}$-depleted cells treated with TX-CPT recovered by 30% (Figure 5B). These results indicate that $p21^{Cip1/WAF1}$ may be required for cytotoxicity to HCT116 cells treated by the TX-CPT regimen.

The induction of $p21^{Cip1/WAF1}$ protein in HCT116 cells treated with TX-CPT regimen through the p38 MAPK pathway

Next, we determined the signaling pathway that induces $p21^{Cip1/WAF1}$ protein synthesis in cells exposed to the TX-CPT regimen. We treated cells with several different signal transduction inhibitors such as p38 mitogen-activated protein kinase (MAPK) inhibitor (SB203580), c-Jun N-terminal kinase (JNK) inhibitor (SP600125) or Chk2 inhibitor. As shown in Figure 6A, cells exposed to p38 inhibitor in the presence of TX-CPT failed to synthesize $p21^{Cip1/WAF1}$, whereas we could not detect a difference in $p21^{Cip1/WAF1}$ levels in cells treated with JNK inhibitor and Chk2 inhibitor, suggesting that in TX-CPT–treated cells, the p38 MAPK pathway regulates $p21^{Cip1/WAF1}$ expression. Finally, we determined whether p38 MAPK is critical to induce cell death in cells exposed to the TX-CPT regimen. In the absence of the p38 inhibitor, cell proliferation decreased 60% (Figure 6B). However, cells exposed to the p38 inhibitor showed a 20% reduction in proliferation, suggesting that the p38 inhibitor reduced TX-CPT-dependent cytotoxicity. Therefore, these results indicate that the TX-CPT induced the expression of $p21^{Cip1/WAF1}$ and enhanced the cytotoxic effect through the p38 MAPK pathway.

Discussion

To date, combinations of CPT and microtubule interfering agents have been tested in a limited number of preclinical studies and have been frequently found to be nonsynergistic or even antagonistic (Kaufmann et al. 1996; Borbe et al. 1999; Schmidt et al. 1999). In colon cancer there is a report showing that the CPT and taxol

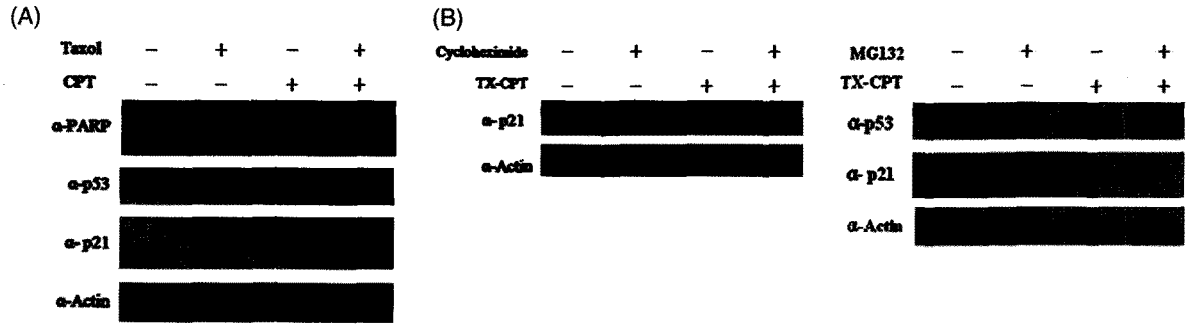

Figure 4. Induction of $p21^{Cip1/WAF1}$ in the HCT116 cells exposed sequentially to taxol and CPT. (A) The expression of PARP, p53 and $p21^{Cip1/WAF1}$ proteins in cells exposed to taxol alone, CPT alone, or the TX-CPT regimen was analyzed by Western blot analysis. β-Actin was used as a loading control. (B) Cells in the TX-CPT regimen were treated with cycloheximide (180 μM) or MG-132 (10 μM). Western blot was performed using anti-p21 and p53 antibodies.

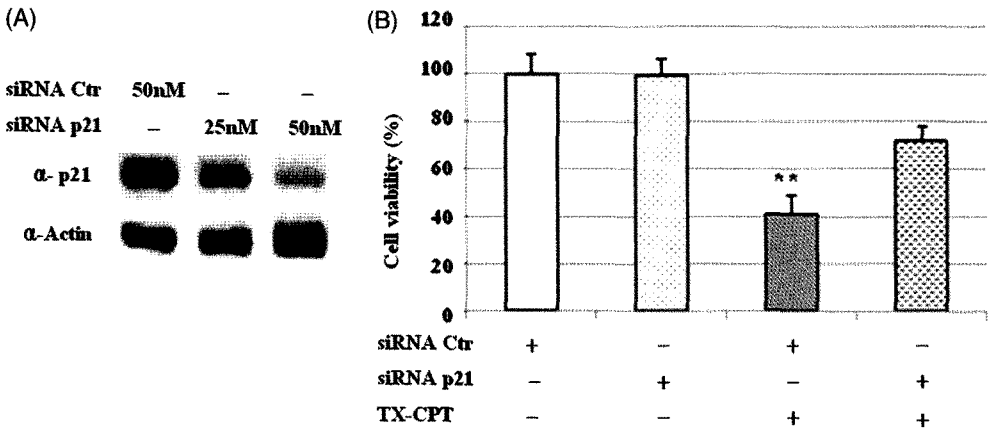

Figure 5. The $p21^{Cip1/WAF1}$ protein is required for the induction of cytotoxicity to cells exposed to the TX-CPT regimen. (A) Transient knockdown of $p21^{Cip1/WAF1}$ protein expression in HCT116 cells using the siRNA approach. (B) After the depletion of $p21^{Cip1/WAF1}$, cell viability was measured in cells treated with the TX-CPT regimen. Data represent the average of three independent experiments. Error bars represent standard error. ** $P < 0.01$ vs. cells transfected with siRNA p21.

combinations resulted in synergistic anticancer activity when these drugs were administered sequentially (Bahadori et al. 2001). However, these previously published studies administered high concentrations of both taxol and CPT compared to our study. Therefore, our study suggests that low-dose anticancer therapeutics can exert similar cytotoxic effects. These effects may occur by taxol-dependent increases in topoisomerase I activity, induced by mitotic arrest, prior to the addition of CPT (Bahadori et al. 2001). This raises the efficacy of the sequentially administered topoisomerase inhibitor CPT in blocking cell cycle progression and affecting cellular stability (Downes et al. 1991). Although the low concentration of CPT (20 nM) causes G1 phase arrest by blocking the DNA topoisomerase complex in asynchronized cells (Pommier et al. 2003), we showed that mitotic cells arrested by taxol showed enhanced cytotoxicity after subsequent CPT treatment. Since topoisomerase is required for chromatid separation, topoisomerase inhibitor CPT catastrophically blocks the transition from metaphase to anaphase (Downes et al. 1991; Shamu and Murray 1992).

In the present study, sequential TX-CPT combinational chemotherapy was cytotoxic to HCT116 cells through the $p21^{Cip1/WAF1}$ protein. Specifically, the level

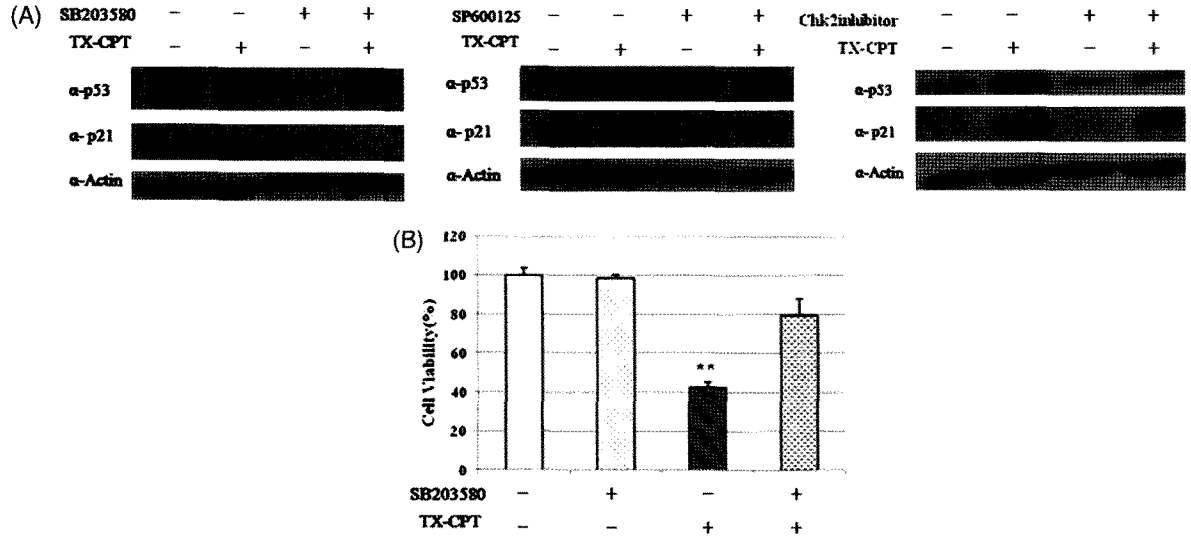

Figure 6. Effect of p38 MAPK inhibitor treatment on the expression of $p21^{Cip1/WAF1}$ in cells sequentially exposed to taxol and CPT. (A) HCT116 cells were pretreated with SB203580 (p38 MAPK inhibitor), SP600125 (JNK inhibitor) and Chk2 inhibitor prior to incubation with the TX-CPT treatment. Western blot analysis was performed with antibodies specific for p53 and $p21^{Cip1/WAF1}$. β-Actin was used as a loading control. (B) Cell viability was determined by the trypan blue exclusion assay. Data represent the average of three independent experiments. Error bars represent standard error. ** $P < 0.01$ vs. cells treated with SB203580.

of $p21^{Cip1/WAF1}$ in TX-CPT-treated cells was increased by synergistic drug interaction. Through knockdown experiments, we confirmed that $p21^{Cip1/WAF1}$ is required for cellular cytotoxicity in cells exposed to the TX-CTP combination. However, our data are not consistent with previous reports insisting that the overexpression of $p21^{Cip1/WAF1}$ reduces cytotoxicity to anticancer drug (Gupta et al. 1997; Furuta et al. 2006). One reason for the difference in results is the cell cycle phase required for $p21^{Cip1/WAF1}$ induction. Since $p21^{Cip1/WAF1}$ protein is expressed in G1 or G2, anticancer drug-dependent cytotoxicity is weak (Gupta et al. 1997; Furuta et al. 2006), whereas $p21^{Cip1/WAF1}$ expression at mitosis directly leads to drug-dependent cell death. Additionally, several studies support the hypothesis that $p21^{Cip1/WAF1}$ overexpression affects cellular cytotoxicity (Chen et al. 1995; Yang et al. 1995). For example, drug sensitivity is increased by $p21^{Cip1/WAF1}$ regardless of p53 (Sinicrope et al. 1998; Satou et al. 2003). The role of $p21^{Cip1/WAF1}$ in cellular anticancer drug effects is dependent on the stages of the cell cycle.

In the present study, the increased $p21^{Cip1/WAF1}$ level following the TX-CPT regimen occurred by the p38 MAPK pathway and not the JNK pathway. Chemotherapeutic drug-induced mitotic arrest, including taxol, induces the enhanced expression of $p21^{Cip1/WAF1}$ through the activation of the p38 MAPK pathway (Bacus et al. 2001; Deacon et al. 2003), supporting our findings regarding taxol in the TX-CPT regimen-treated cells. $p21^{Cip1/WAF1}$ is influenced by the p38 MAPK pathway in response to exogenous stress such as nitric oxide (Ciccarelli et al. 2005; Cui et al. 2005). Therefore, in the TX-CPT regimen, taxol and sequential CPT treatment resulted in p38 MAPK pathway activation, thereby inducing $p21^{Cip1/WAF1}$ protein synthesis (Han et al. 2002; Satou et al. 2003).

In contrast to the TX-CPT regimen, the CPT-TX regimen did not affect cell death in HCT116 cells. CPT first induces G1 arrest by activating several repair checkpoints (Sinicrope et al. 1998; Satou et al. 2003). A subsequent low-dose taxol treatment is unable to affect microtubule polymerization in the M phase. This was similar to our observations of HCT116 cells simultaneously treated with CPT and taxol, as well as CPT alone, leading further support to the TX-CPT regimen as an optimal combination for chemotherapy.

Taken together, our results suggest that, in the TX-CPT regimen, taxol acts to sensitize the subsequent CPT-exposed cells to enhance cytotoxicity. This is similar to the role of taxol prior to irradiation as a synergistic cancer therapy (Tishler et al. 1992; Liebmann et al. 1994). Like our strategy, the low concentration of anticancer agents targeting different pathways reduces adverse effects that arise from chemotherapy, which is a limiting factor for efficient cancer therapy. Our results could provide critical insights into which human tumors are eliminated efficiently and exclusively through therapeutic intervention. Further studies on preclinical cancer effects will be required to verify whether the combination of taxol and CPT can be used for effective cancer therapy.

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