Chk1 and Chk2 are differentially involved in homologous recombination repair and cell cycle arrest in response to DNA double-strand breaks induced by camptothecins

Min Huang,[1] Ze-Hong Miao,[1] Hong Zhu,[1] Yu-Jun Cai,[1] Wei Lu,[2] and Jian Ding[1]

Divisions of [1]Anti-tumor Pharmacology and [2]Chemistry, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, Peoples' Republic of China

Abstract

Camptothecins (CPT) activate S or G_2-M arrest and the homologous recombination (HR) repair pathway in tumor cells. In this process, both checkpoint kinases 1 and 2 (Chk1 and Chk2, respectively) are activated, but their differential roles, especially in the coordination of checkpoint and repair control, and potential clinic relevance remain to be fully elucidated. In this study, the repairable double-strand breaks were induced in human colon cancer HCT116 cells by 1-h exposure to 25 or 100 nmol/L CPT and its novel derivative chimmitecan. The cellular disposal of double-strand breaks was reflected as the progressive dispersal of γ-H2AX foci, reduction of ''comet'' tails, dynamic activation of RAD51-mediated HR repair, and reversible G_2-M arrest. In this model, the differential kinetics of Chk1 and Chk2 activation was characterized by the progressively increased phosphorylation of Chk2 until 72 h, the degradation of Chk1, and the disappearance of phosphorylated Chk1 48 h after drug removal. Using RNA interference, we further showed that Chk2 was essential to G_2-M arrest, whereas Chk1 was mainly required for HR repair in CPT-treated HCT116 cells. Moreover, Chk2, rather than Chk1, predominated over the control of cell survival in this model. The differential roles of Chk1 and Chk2 in regulating HR repair and G_2-M phase arrest were also confirmed in HT-29 colon cancer cells. Together, these findings systematically dissect the differential roles of Chk1 and Chk2 in a favorable model pursuing CPT-driven DNA damage responses, providing critical evidence to further explore checkpoint modulation, especially Chk2 inhibition as a therapeutic strategy in combination with CPT. [Mol Cancer Ther 2008;7(6):1440–9]

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Requests for reprints: Jian Ding, Division of Anti-tumor Pharmacology, State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zu Chong Zhi Road, Zhangjiang Hi-Tech Park, Shanghai 201203, Peoples' Republic of China. Phone: 86-21-50806722; Fax: 86-21-50806722. E-mail: jding@mail.shcnc.ac.cn

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Introduction

Camptothecin (CPT), whose derivatives are among the most effective anticancer drugs, is a specific topoisomerase I poison. A novel CPT derivative, chimmitecan, has been shown to possess improved anticancer potency and pharmacologic profiles, compared with the clinically available CPT analogues, and is moving forward as an anticancer agent candidate (1). CPT trap topoisomerase I-DNA cleavable complexes and cause replication-dependent DNA double-strand breaks (DSB; ref. 2), which have been revealed to trigger S and G_2-M arrest (3, 4) and simultaneously to be repaired mainly through the homologous recombination (HR) repair pathway (5). The anticancer potency and selectivity of CPT is accordingly recognized to be closely related to tumor-specific defects in cell cycle checkpoint and DNA repair (6).

The two structurally unrelated but functionally similar protein serine/threonine kinases, checkpoint kinases 1 and 2 (Chk1 and Chk2, respectively), play critical roles in cell cycle arrest driven by CPT (7, 8). Both kinases are known to relay the checkpoint signals from the upstream signal-transducing kinases of the phosphatidylinositol kinase-related family, particularly the ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and RAD3 (ATR) related to the proximal substrates (9). Chk1 is known to be activated by phosphorylation at Ser^{345} or Ser^{317}, in turn phosphorylate Cdc25A/C, and ultimately arrest cells in late S or G_2 phases (10, 11). The activation of Chk2 in checkpoint signaling is initiated by phosphorylation at Thr^{68} in an ATM–dependent manner (12). In addition to checkpoint regulation, recent discoveries have highlighted the expanded roles of both kinases in controlling DNA repair, genomic stability, and apoptosis (13–15). For instance, Chk1 and Chk2 regulate DSB repair by phosphorylating the repair factors RAD51 (16) or BRCA1 (17), respectively. The overlapping substrates of Chk1 and Chk2, such as cell cycle regulation proteins Cdc25A/C, p53, and Mdm2 (13), may underlie their redundant roles in damage response. Notably, increasing evidence indicates that Chk1 and Chk2 have respective specific substrates and thereby exert differential cellular functions. However, these differential functions of the two kinases, particularly in response to DSB triggered by CPT, remain to be characterized in detail.

Furthermore, inhibitors of Chk1 and Chk2 have been shown to potentiate the therapeutic efficacy of DNA-damaging agents, including CPT, and proposed as adjuvant

therapeutics to circumvent tumor resistance to anticancer drugs (18). Their nonselective inhibitor UCN-01 is undergoing clinical evaluations either alone or in combination with established cytotoxic agents (7, 19). A series of specific inhibitors are in preclinical development (20, 21). Nevertheless, which kind of these inhibitors, for Chk1, for Chk2, or for both, will be better for clinical cancer therapy remains be fully elucidated. Demonstration of the differential functions of the two kinases in DNA repair and cell cycle control may clarify this question and thus forcefully put forward the development of their inhibitors as new anticancer therapeutics.

In this study, we generated a cellular model that dynamically reflected the repair of DSB, cell cycle arrest, and its recovery in response to CPT and its new derivative chimmitecan in human colon cancer HCT116 and HT-29 cells. Using this model, we investigated the roles of Chk1 and Chk2 in regulating HR repair and cell cycle arrest by monitoring their activation and specific requirements for both pathways. Our results uncover the respectively predominant roles of Chk1 and Chk2 in the disposal of CPT-driven DSB. Further, our findings suggest that the combination of CPT with Chk2 inhibitors is likely to be a better strategy in cancer chemotherapy.

Materials and Methods

Materials

Chimmitecan was synthesized by Chemistry Department of Shanghai Institute of Materia Medica, Chinese Academy of Sciences (1). The purity was 98.6% as determined by high-performance liquid chromatography. CPT was purchased from Sigma. Both compounds were dissolved at 10 mmol/L in DMSO (Sigma) and stored at -20°C. Dilutions of the compounds were made up in normal saline before each experiment. Anti-γH2AX, p-Chk1, Chk2, and p-Chk2 antibodies were purchased from Cell Signaling Technology. Antibodies for Chk1, p-Cdc2, Cdc2, cyclin B1, and glyceraldehyde-3-phosphate dehydrogenase were obtained from Santa Cruz Biotechnology. The secondary antibody conjugated with Alexa 488 was purchased from Molecular Probes.

Cell Culture and Drug Treatment

Human colorectal carcinoma HCT116 and HT-29 cells were purchased from the American Type Culture Collection. The cells were maintained in McCoy's medium (Sigma) supplemented with 20% fetal bovine serum (10% for HT-29; Life Technologies), gentamycin (2.5 μg/mL), and HEPES (10 mmol/L; pH 7.4) in a humidified atmosphere of 95% air/5% CO_2 at 37°C. In all experiments involving drug treatments, the cells were treated with CPT or chimmitecan for 1 h, washed with PBS, and incubated in drug-free medium for the indicated time.

Flow Cytometry

Cell cycle distribution was measured using flow cytometry (FACSCalibur; Becton Dickinson) after standard fixation and propidium iodide staining and analyzed with CellQuest and ModFit LT3.0 software (Becton Dickinson). The detection of DNA synthesis in S phase was done using Bromodeoxyuridine Flow Kit (BD PharMingen). The Annexin V and propidium iodide dual labeling of apoptotic cells was conducted using the Annexin V-FLUOS Staining Kit from Roche Applied Science according to the manufacturer's instructions.

Neutral Comet Assay

Neutral comet assay was applied to detect DSB. After exposure to CPT for 1 h, cells embedded in agarose were lysed and subjected to electrophoresis as described previously (22). Individual cells stained with 4′,6-diamidino-2-phenylindole (0.5 μg/mL) were viewed using a UV light fluorescence microscope (Olympus). Quantification was achieved by analyzing 50 randomly selected comets per slide with Komet 5.5 software (Kinetic Imaging) using the variable Olive Tail Moment (arbitrary units; defined as the product of the percentage of DNA in the tail multiplied by the tail length).

Small Interfering RNA Transfection

Chk1, Chk2, and control (green fluorescence protein) small interfering RNA (siRNA), as described previously (23–25), were synthesized by Shanghai GenePharma. Four pairs of RAD51 siRNA were designed, with the most efficient one (5′-GGCGGUCAGAGAUCAUACAdTdT-3′) applied in this study. HCT116 cells were transfected with Oligofectamine reagent (Invetrogen) according to the manufacturer's instruction. The final concentration of all siRNA was at 100 nmol/L. Drug treatment was done 24 h (48 h for HT-29 cells) after transfection.

Immunocytochemistry

Drug-treated cells were fixed with 4% paraformaldehyde and permeablized with 0.2% Triton X-100 before being blocked with PBS containing 3% bovine serum albumin. γ-H2AX foci were detected by anti-γ-H2AX primary antibody and visualized using Alexa 488–conjugated secondary antibody. For RAD51 foci detection, the cells were treated with extraction buffer [0.5% Triton-X100 in 200 mmol/L HEPES (pH 7.4), 50 mmol/L NaCl, 3 mmol/L $MgCl_2$, 300 mmol/L sucrose] on ice for 15 min before fixation with 4% paraformaldehyde (16). After staining with 4′,6-diamidino-2-phenylindole (0.5 μg/mL), the images were photographed using a Leica TCS confocal microscope (Leica Microsystems). The relative intensity of RAD51 and γ-H2AX foci at each time point was measured by calculation of the ratio of the green signal intensity (RAD51/γ-H2AX foci) to blue signal (4′,6-diamidino-2-phenylindole/nucleus), with the relative intensity at time 0 h (for γ-H2AX) or 6 h (for RAD51) being defined as 1, respectively. Data are expressed as mean ± SD of three independent experiments.

Western Blot Analysis

CPT-treated cells were lysed and Western blot analysis was done following routine process as described previously (26). Semiquantification of γ-H2AX expression was applied by densitometric analysis. Relative γ-H2AX level was calculated by the ratio of γ-H2AX expression relative to glyceraldehyde-3-phosphate dehydrogenase expression. Data are expressed as mean ± SD of three independent experiments.

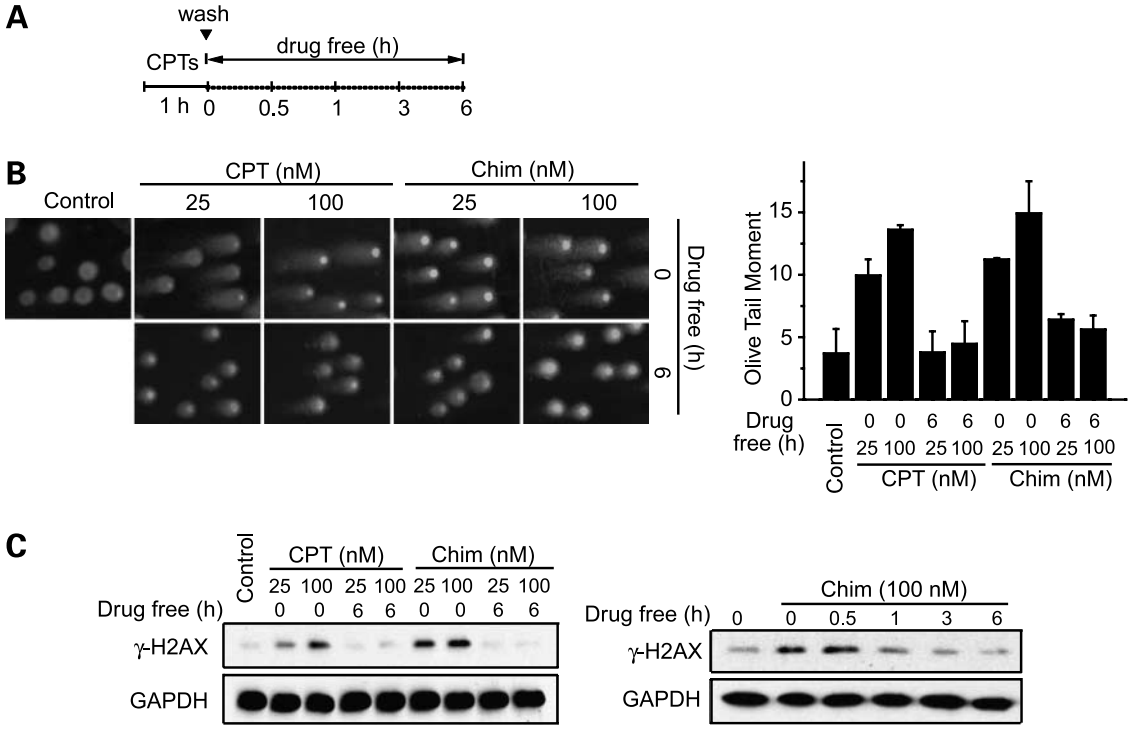


Figure 1. Elimination of DSB induced by CPT within 6 h after drug treatment. **A,** procedure for drug treatment. HCT116 cells were treated with CPT or chimmitecan at 25 or 100 nmol/L for 1 h. Immediately after removal of drugs (at 0 h), cells were washed with PBS and incubated in drug-free medium for the indicated time. **B,** representative images of neutral comet assays and semiquantification of DSB (see Materials and Methods). *Columns,* mean of three independent experiments; *bars,* SD. **C,** Western blot analyses of progressive γ-H2AX attenuation after drug removal, with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the loading control. *Chim,* chimmitecan.

Statistical Analysis

Data were presented as mean ± SD, and differences were considered significant when P value was <0.05 as determined by Student's t test.

Results

DNA Repair and Cell Cycle Arrest following CPT-Elicited DSB

To establish a reliable cellular model to distinguish the roles of Chk1 and Chk2 in DNA repair and cell cycle arrest, we treated HCT116 cells for 1 h with CPT followed by drug-free incubation for different durations (Fig. 1A). The neutral comet assay (27) and the histone H2AX phosphorylation (γ-H2AX) assay (28) were employed to survey DNA repair progression, and flow cytometry was used to monitor the cell cycle progression. One-hour exposure of HCT116 cells to both CPT and chimmitecan resulted in prominently concentration-dependent DSB. At the 6-h time point after drug removal, detectable DSB were considerably reduced, indicative of the rapid DSB elimination (Fig. 1B and C). In contrast to such fast religation of DSB, the recovery of cell cycle progression from arrest experienced a relatively slow process. Time-course flow cytometry revealed an instant intra-S-phase replication blockade and successive persistent G_2-M arrest until 24 or 48 h following 1-h exposure to 25 nmol/L CPT, consistent with previous findings (4). Most arrested cells were finally released from checkpoint arrest and recovered to normal progression after DSB elimination, marked by regular cell cycle distribution and seldom sub-G_1 apoptotic cells at 48 or 72 h after drug removal (Fig. 2A-C). Similar results were achieved at 100 nmol/L (data not shown). Therefore, we set up the conditions of repairable DSB and recoverable cell cycle arrest in HCT116 cells. It will be used to define the roles of Chk1 and Chk2 in the following sections.

For further confirmation, we also detected CPT and chimmitecan-triggered responses using another colon cancer HT-29 cell line. Our results showed a similar reversible G_2-M-phase arrest (Supplementary Fig. S2)[3] but a slowed DSB disposal over 12-h drug-free incubation (Supplementary Fig. S1A).[3] It was noticed that equal concentration of CPT exerted weaker potency in inducing cell cycle arrest in HT-29 cells compared with HCT116 cells, which may attribute to their defective in p53-14-3-3σ pathway (29). Further, the slowed DSB removal in HT-29 cells may implicate the crucial requirement of substantial cycle arrest in DNA repair.

[3] Supplementary material for this article is available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Efficient Elimination of CPT-Elicited DSB by RAD51-Mediated HR Repair Pathway

To determine the roles of Chk1 and Chk2 in DNA repair, we attempted to detect which repair pathway was employed in the current model to repair the DSB elicited by CPT. Previous findings have shown that CPT-induced DSB are predominantly repaired by HR repair in mammalian cells, in which the essential protein RAD51 is detected as foci in multiple discrete subnuclear structures (5, 30). Immunofluorescence analyses revealed the quick and persistent activation of HR repair, manifested as the kinetics of the RAD51 foci formation. Meanwhile, CPT-stimulated γ-H2AX foci gradually scattered and almost disappeared at 6 h (Fig. 3A). The good dynamic correlation between RAD51 activation and DSB removal suggested that the instant activation of the RAD51-mediated HR repair pathway was probably responsible for the rapid elimination of DSB in HCT116 cells. Further, transfection of specific RAD51 siRNA into cells successfully knocked down the RAD51 protein expression (Fig. 3C; Supplementary Fig. S1B)[3] and, as expected, effectively prevented CPT-induced DSB from being repaired (Fig. 3B-D). Accordingly, RAD51 disruption also slowed down the recovery of the arrested cell cycle progression (Fig. 3E), which helped to show the requirement of proficient repair in cell cycle progression. It was also noticed that transfection with RAD51 siRNA induced the appearance of sub-G_1 population at 24, 48, and 72 h (Fig. 3E), which highlighted the potential role of RAD51-mediated HR repair pathway in the repair of CPT-induced damage in HCT116 cells. These data collectively confirm that the HR repair pathway is mainly responsible for repairing the DSB caused by CPT.

Differential Activation Kinetics of Chk1 and Chk2 in CPT-Treated HCT116 Cells

To reveal the differences between Chk1 and Chk2, we firstly used the established model to characterize the activation kinetics of Chk1 and Chk2 in CPT-treated HCT116 cells. As expected, Chk1 was phosphorylated instantly after 1-h exposure to 25 nmol/L chimmitecan and Chk2 was activated a little later, at 3-h time point following drug treatment. The unexpected was that they were activated in distinct kinetics. The phosphorylation of Chk1 at Ser^{317} and Ser^{345} was enhanced until 24 h after CPT withdrawal and then disappeared after 48 h. The decline of phosphorylated Chk1 at the later stage was likely attributed to its proteolytic degradation initiated at 48 h (Fig. 4A; ref. 31). In contrast, Chk2 showed a trend of sustained activation indicated by progressively increased

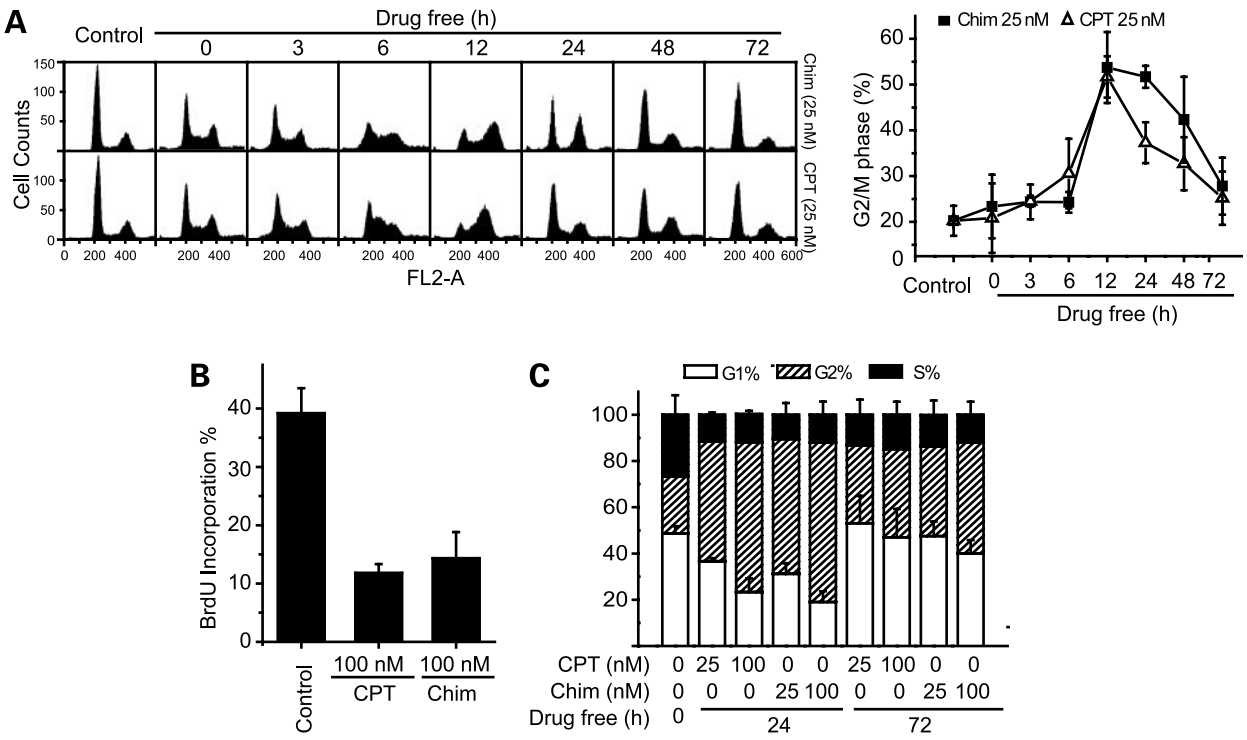


Figure 2. Reversible G_2-M-phase arrest induced by CPT. **A,** time course of cell cycle arrest induced by CPT. HCT116 cells were treated as described in Fig. 1A, harvested at the indicated time points, and then subjected to flow cytometry. **B,** DNA synthesis inhibition by CPT. Cells were labeled with 10 μmol/L bromodeoxyuridine for 60 min at the end of 6-h incubation as described in Fig. 1A and subjected to fluorescence-activated cell sorting analyses. Bromodeoxyuridine-positive cells were counted and expressed as percentage in relative to total cell population. **C,** CPT-induced reversible G_2-M phase arrest. Cells were collected at the end of 24- or 72-h incubation and G_2-M-phase cells were measured based on DNA content by flow cytometry. *Columns,* mean of three independent experiments; *bars,* SD.

phosphorylation at Thr[68] until 72 h. The differential activation kinetics of Chk1 and Chk2 suggests their distinct roles in DSB elicited responses in HCT116 cells. In addition, the levels of the G_2-M transition-related proteins, like phosphorylated Cdc2 and cyclin B1, increased as the cell cycle was arrested and returned to normal as the cell cycle progression recovered (Figs. 2A and 4A).

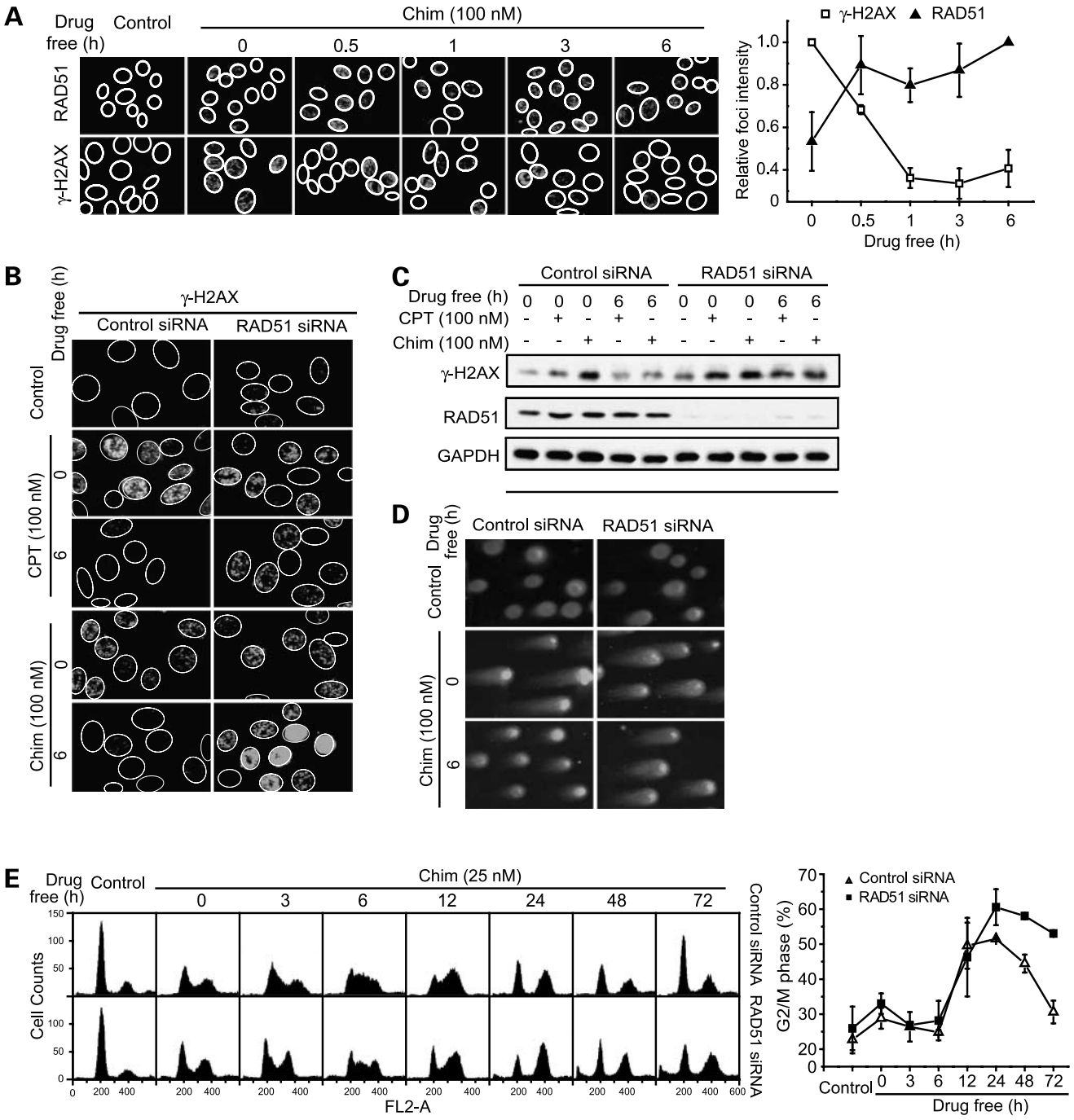


Figure 3. Requirement of RAD51-mediated HR repair for DSB attenuation and cell cycle recovery following CPT treatment. Cells were treated (as described in Fig. 1A) 24 h after transfection with RAD51 or control (green fluorescence protein) siRNA. **A,** kinetics of RAD51 foci formation and γ-H2AX foci dispersal in cells following drug removal. Nuclear γ-H2AX and RAD51 foci were detected by immunostaining. *White circles,* nuclei. Relative intensity of RAD51 and γ-H2AX foci was calculated as described in Materials and Methods. **B,** immunofluorescence of CPT-induced γ-H2AX foci. **C,** Western blot analyses of γ-H2AX levels. **D,** neutral comet analyses of DSB. **E,** kinetics of chimmitecan-induced cell cycle arrest following RAD51 or control siRNA transfection. Cell cycle distribution was determined by flow cytometry. *Points,* mean of three independent experiments; *bars,* SD.

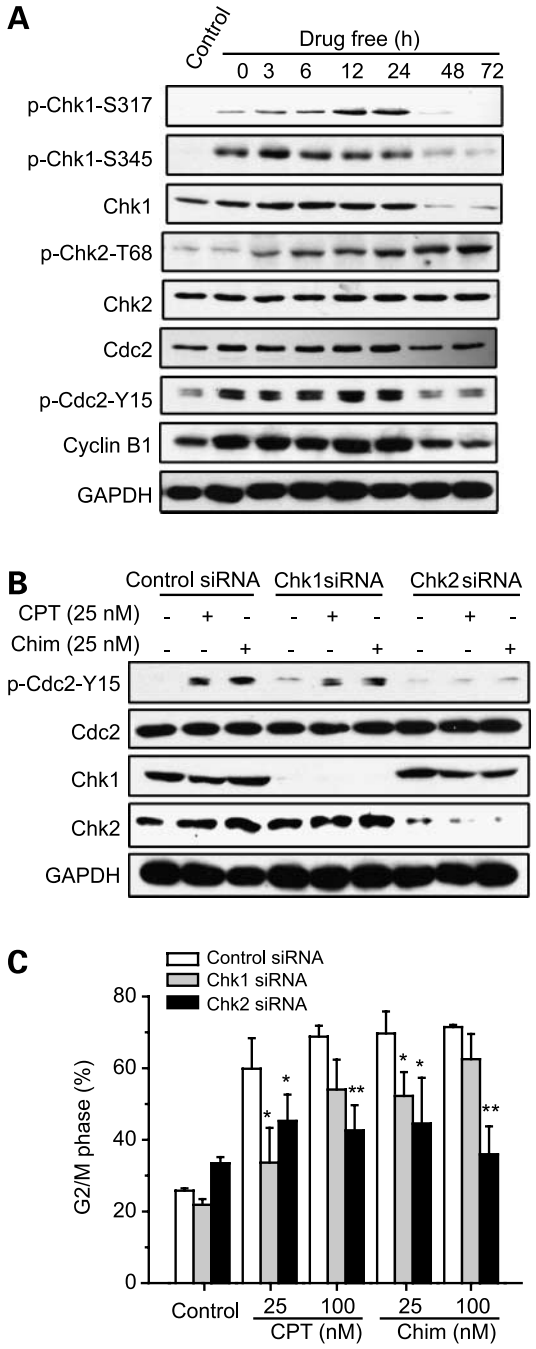


Figure 4. Dynamic activation of Chk1 and Chk2 following chimmitecan treatment and their individual involvements in CPT induced G_2-M checkpoints. **A,** HCT116 cells were exposed to 25 nmol/L chimmitecan as described in Fig. 1A, harvested, and lysed at the indicated time points. Protein concentrations were determined using a bicinchoninic acid protein assay kit before subjecting to Western blot analyses. **B,** effects of Chk1 and Chk2 siRNA on CPT-induced phosphorylation of Cdc2. HCT116 cells were treated as described in Fig. 1A 24 h after transfection with Chk1, Chk2, or control siRNA, respectively. Western blot analyses were done after 6-h incubation. **C,** effects of Chk1 and Chk2 siRNA on CPT-induced G_2-M phase arrest. After 24-h incubation in drug-free medium, cells were subjected to flow cytometry for cell cycle analyses. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$, Chk1 or Chk2 versus green fluorescence protein siRNA-transfected group.

Differential Requirements of Chk1 and Chk2 in CPT-Induced G2-M Arrest

To dissect the individual contributions of Chk1 and Chk2 to arresting cells at G_2-M transition, HCT116 cells were transfected with specific siRNA targeting Chk1 or Chk2 24 h before exposure to 25 and 100 nmol/L CPT or chimmitecan. Western blotting showed that the siRNA transfection dramatically reduced the corresponding protein expression (Fig. 4B; Supplementary Fig. S3A).[3] The subsequent flow cytometry analyses in both HCT116 cells (Fig. 4C) and HT-29 cells (Supplementary Fig. S3B)[3] revealed that the disruption of either Chk1 or Chk2 reduced the cells arrested in G_2-M phase compared with the reference group transfected with green fluorescence protein siRNA. However, the degree of Chk1 or Chk2 siRNA-caused reduction in G_2-M arrested cells was different. The Chk2 siRNA elicited more prominent decrease in the cells accumulated in G_2-M phase in response to CPT treatment than Chk1 siRNA (Fig. 4C). Consistently, Chk2 depletion almost completely eliminated CPT-induced increase in phosphorylated Cdc2 (at Tyr15; Fig. 4B), the crucial mitotic cyclin-dependent kinase (18). In contrast, Chk1 siRNA exerted relatively minor effects on both the phosphorylation of Cdc2 and the release of the cells from the CPT-induced G_2-M arrest (Fig. 4B and C). These results indicate that Chk2, rather than Chk1, is essential to the control of CPT-induced G_2-M arrest, although we cannot rule out their both involvements.

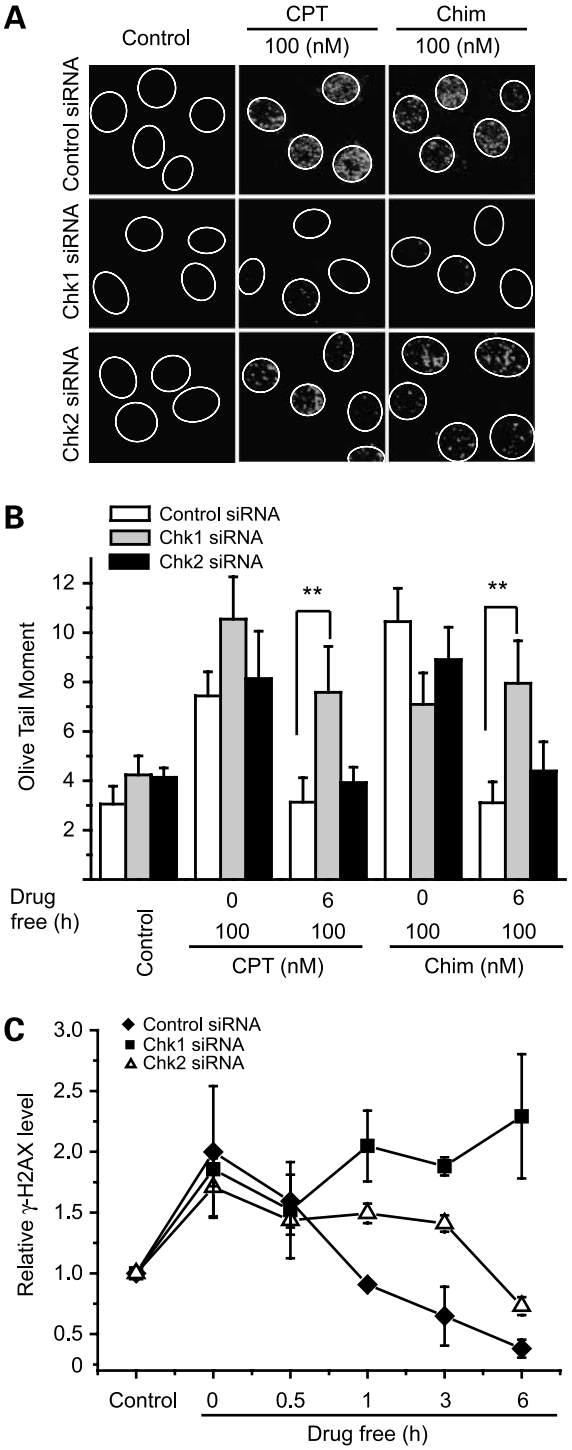
Distinct Roles of Chk1 and Chk2 in CPT-Activated HR Repair

In the current model, cells could effectively repair both CPT- and chimmitecan-induced DSB through initiating the HR repair mechanism (Figs. 1 and 3). We next investigated the respective engagement of Chk1 and Chk2 in this repair pathway. Interference with Chk1 considerably prevented the formation of RAD51 foci following the treatment with CPT or chimmitecan (Fig. 5A), which led to high levels of the unrepaired DSB persisting over 6 h after drug removal (Fig. 5B and C). At that time, however, the corresponding DNA damages were efficiently repaired in Chk1-proficient cells (Figs. 1 and 3). In contrast, the disrupted the expression of Chk2 with its specific siRNA just slightly delayed the process of DNA repair (Fig. 5B and C). Similarly, Chk1 siRNA exerted greater effect than Chk2 siRNA in diminishing CPT triggered RAD51 foci formation in HT-29 cells (Supplementary Fig. S4).[3] These data highlight the requirement of Chk1 rather than Chk2 for the repair of DSB by HR repair pathway after CPT treatment.

Predominant Role of Chk2 versus Chk1 in Sustaining Cell Viability in HCT116 Cells

From the results above, we have successfully defined the differences between Chk1 and Chk2 in controlling HR repair and G_2-M-phase arrest triggered by CPT and chimmitecan. We finally detected whether such differences contributed to cell death induced by CPT in hopes of a better understanding of the endpoint events driven by Chk1- or Chk2-generated defective repair or cycle arrest.

Annexin V and propidium iodide dual-labeling analyses showed that few cells underwent apoptosis 24 h after exposure to 100 nmol/L CPT. However, the depletion of Chk2, rather than Chk1, obviously increased the apoptotic cell population induced by CPT (Fig. 6A). Consistently, Chk2 but not Chk1 siRNA dramatically enhanced the proportion of sub-G_1 apoptotic cells 72 h after drug removal (Fig. 6B). We therefore conclude that Chk2 is more important than Chk1 in sustaining cells survival in the DNA damage stress induced by CPT.



Discussion

Chk1 and Chk2, both mediators in DNA damage response, have been shown to share some overlapping substrates and possess redundant functions. They can phosphorylate common substrates such as Cdc25C and p53 (13, 32, 33), control cell cycle progression (8, 34), regulate DNA repair (16, 35), and coordinate cell survival and death (14, 36). Meanwhile, the two kinases display obvious distinction in stability and activation patterns responding to genotoxic stress (13). More importantly, the biological requirements for Chk1 and Chk2 functions are strikingly different, as Chk1 but not Chk2 is essential for embryonic development and viability (37, 38). Therefore, it is fundamentally important to delineate their differential functions in DNA damage response regulation.

For this purpose, we established a stable experimental model by using CPT and/or its new derivative chimmitecan (1) to treat colon cancer cells for 1 h and then incubating the cells in drug-free medium for different durations. This model dynamically reflects DNA repair, cell cycle arrest, and its recovery in response to the insults of CPT. Using this model, we uncovered the differential kinetics of Chk1 and Chk2 activation, characterized by the progressively increased phosphorylation of Chk2 at Thr^{68} until 72 h, the degradation of Chk1, and the disappearance of its phosphorylated form 48 h after drug removal. With the aid of the RNA interfering technology, we further showed that Chk2 was essential to the G_2-M arrest, whereas Chk1 was mainly required for the HR repair in CPT-treated HCT116 and HT-29 cells. All these differences, herein systematically revealed for the first time, manifest the differential roles of Chk1 and Chk2 in several critical cellular activities like DNA repair and cell cycle progression following DNA damage.

Phosphorylated activation of Chk1 is mainly dependent on ATR, whereas Chk2 is preferred to be activated by ATM.

Figure 5. Roles of Chk1 and Chk2 in CPT-induced HR repair. HCT116 cells were treated as described in Fig. 1A 24 h after transfected with Chk1, Chk2, or control siRNA, respectively. **A,** effects of Chk1 and Chk2 siRNA on CPT-activated HR repair. RAD51 foci were detected by immunofluorescence at the end of 6-h incubation in drug-free medium. *White circles,* nuclei. **B,** repair efficiency was evaluated by neutral comet assays indicated by Olive Tail Moment (see Materials and Methods). **, $P < 0.01$, Chk1 or Chk2 versus green fluorescence protein siRNA-transfected group. **C,** effects of Chk1 and Chk2 siRNA on kinetics of DSB removal. Remaining DSB were measured according to the γ-H2AX levels determined by Western blot analyses. Semiquantification of γ-H2AX expression was applied by densitometric analyses. Relative γ-H2AX levels were calculated as the ratio of γ-H2AX expression relative to that of glyceraldehyde-3-phosphate dehydrogenase. *Columns,* mean of three independent experiments; *bars,* SD.

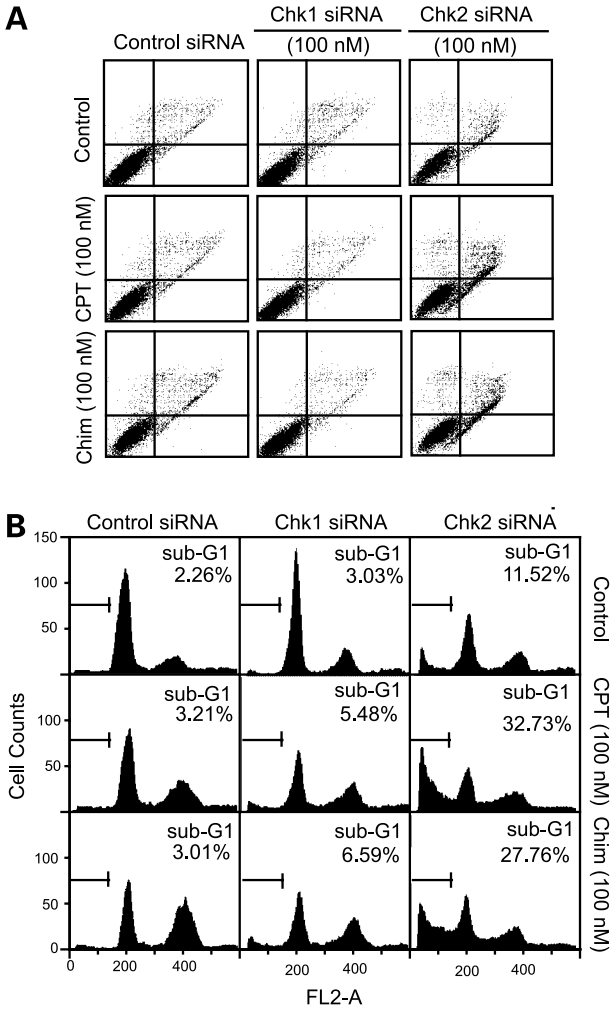


Figure 6. Roles of Chk1 and Chk2 in CPT-induced apoptosis. HCT116 cells were treated as described in Fig. 1A 24 h after transfected with Chk1, Chk2, or control siRNA, respectively. **A,** at the end of 24-h incubation in drug-free medium, cells were dual labeled with Annexin V and propidium iodide before flow cytometry analyses. **B,** cells were incubated in drug-free medium for 72 h following CPT treatment. Apoptotic cells were specified by sub-G_1 cells with flow cytometry.

ATR related-Chk1 pathway is usually activated by blocked replication fork progression, whereas the ATM-Chk2 pathway responds to DSB (33, 39). CPT traps topoisomerase I-DNA cleavage complexes, which collide with and block the progressing replication forks, leading to replication DSB (2). Thus, both Chk1 and Chk2 are activated in response to CPT treatment. Despite the overlapping substrates, Chk1 and Chk2 have their respective unique substrates (13), which may explain the differential roles of Chk1 and Chk2 in our experimental models. In addition, such differential roles could be associated with the early degradation (Fig. 4; ref. 4) and retreat of Chk1 from the ongoing active damage signaling, which could up-regulate the Chk2 pathway in a complementary manner (Fig. 4) as revealed previously (36). Because the cycle response at 72 h is similar to control group (Fig. 3B), the reason of the persisting phosphorylation of Chk2 (Fig. 4A) remains to be elucidated. We propose that Thr^{68} Chk2 may be activated for its other functions, such as the negative regulation of mitotic catastrophe and antiapoptotic effect in response to genetic stress (14, 40). In agreement with this view, the down-regulation of Chk2 failed to inhibit Cdc2, resulting in premature entry into the mitotic phase and subsequent cell death.

To date, the role of Chk2 in DNA damage–caused apoptosis is controversial. Chk2 is conflictingly recognized as both a promoter and an inhibitor of apoptosis. According to our results, Chk2 siRNA dramatically enhanced CPT-induced apoptosis in HCT116 cells, suggesting that Chk2 is likely an apoptosis inhibitor. We propose that Chk2 may circumvent its own tumor-suppressive functions by promoting survival in the current model. Castedo et al. (40) have shown that inhibition of Chk2 reduced doxorubicin-induced G_2 blockade and concomitantly increased the frequency of apoptotic cells in HCT116 cells. Also, Chk2 has been proven to provide a survival signal for tumor cells. Molecular and genetic targeting of Chk1 enhances DNA damage–induced apoptosis (14). Yu et al. also showed that inhibition of Chk2 facilitated CPT-induced apoptosis. All these evidence was consistent with our findings. On the other hand, Chk2 has been regarded as a tumor suppressor, which enhances apoptosis by stabilizing p53 (41–43). It seems that the role of Chk2 varies along with the cellular genetic background, damage forms, and potency. Further efforts are still needed to elucidate the role of Chk2 in DNA damage response.

Given the crucial roles of Chk1 and Chk2 in the DNA-damaging response pathways, modulation of their activity has been proposed to yield adjuvant therapeutics, especially against tumor drug resistance (18). The Chk1 inhibitor UCN-01 has been in phase I and II trials either alone or in combination with cytotoxic agents (7). A more recently released Chk1 inhibitor, Gö6976 is reported to enhance the cytotoxicity of the topoisomerase I inhibitor SN38 in p53-defective cells (20). Meanwhile, selective inhibition of Chk2 is also considered to be a favorable strategy in improving the therapeutic index of CPT and a couple of specific inhibitors have emerged (6, 21, 44). According to our results, Chk2-targeted inhibitors might provide a more promising strategy in combination with CPT, because depletion of Chk2 but not Chk1 dramatically increased the proportion of apoptotic HCT116 cells exposed to CPT. However, Flatten et al. highlighted previously the role of Chk1 in determining the sensitivity to topoisomerase I poisons (45). The different results stress the potentially conditional roles of these kinases in response to CPT insults, because the current study used a 1-h exposure protocol, but Flatten et al. employed a persistent treatment procedure. Our data show that disruption of Chk2 forces tumor cells into apoptosis when exposed to noncytotoxic concentrations of CPT. This is important because Chk2 is not essential for mammalian

viability (13). Thus, the combination of CPT with Chk2 inhibitor(s) in cancer therapy might generate therapeutic potentials but lower risks of systematic toxicity than that of CPT with Chk1 inhibitor(s). Nevertheless, we must point out that these findings may only provide limited clinical relevance, considering that apoptosis may not be the major mode of cell death caused by CPT, in spite of the tremendous evidence revealing CPT-induced apoptosis (46). Moreover, increasing studies have suggested that CPT-treated cells, especially those with defective cell cycle checkpoint, eventually undergo mitotic catastrophe, a type of cell death that occurs during mitosis instead of apoptosis (47, 48). At the current stage, we cannot rule out the possibility that mitotic death is another important way of cell death, because inhibition of Chk2 has been shown to facilitate the induction of mitotic catastrophe in HCT116 cells (40).

In addition, the detailed kinetics of cell cycle arrest and HR repair driven by CPT offers a deeper understanding of the coordination between these two pathways in surveillance of genomic integrity. On occurrence of CPT-caused DSB, both HR repair and cell cycle checkpoints were rapidly and simultaneously activated, evidenced by intra-S-phase arrest and RAD51 foci formation shortly after drug withdrawal. More importantly, the two pathways progressed in an interdependent manner. The HR repair machineries functioned as the cell cycle checkpoints were activated. The initial S-phase delay and subsequent long-term G_2-M arrest jointly evidenced the whole process of DSB repair. The interplay between these two pathways was further confirmed by the fact that depletion of the repair factor RAD51 apparently delayed the cell cycle recovery, whereas the knockdown of the checkpoint kinases disrupted or suppressed the HR repair. Nevertheless, disrupting the two pathways, respectively, does not necessarily impose equal effects on cell viability after CPT treatment. Under our conditions, Chk1 is preferably involved in CPT-stimulated HR repair, whereas Chk2 plays a predominant role in inducing G_2-M-phase arrest and in protecting cells from apoptosis following CPT treatment. It seemed that a greater effect was imposed by checkpoint regulation on cell survival after DSB, in agreement with the recent finding concerning the relative contribution of DNA repair and cell cycle checkpoint in the maintenance of genomic stability (49).

In summary, our study, for the first time, systematically compared the distinct roles of Chk1 and Chk2 in CPT-triggered damage responses, especially in HR repair and cell cycle checkpoints. The reliable model we applied allows us a comprehensive understanding of Chk1 and Chk2 functions responding to CPT treatment. Our data suggest that the two kinases perform in concert to ensure insulted cells to initiate checkpoint signaling and accomplish DNA repair. Specifically, Chk2 plays a predominant role in inducing and maintaining DSB-caused G_2-M arrest, whereas Chk1 distinguishes its role in HR repair regulation, although we cannot rule out their common engagements in both pathways. These results provide critical evidence to further explore the potential of Chk2 modulation in sensitizing tumor cells to CPT treatment.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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