ABSTRACT

7-Hydroxystaurosporine (UCN-01) is a selective protein kinase C inhibitor in clinical trial for cancer treatment. In this study, we found that nanomolar concentrations of camptothecin (CPT), a topoisomerase I inhibitor, arrest or delay cell cycle progression during the S and G2 phases in p53 mutant human colon carcinoma HT29 cells and that UCN-01 abrogates the S-phase arrest or delay induced by CPT. Under these conditions, CPT increased cyclin A levels and cyclin A/cyclin-dependent kinase 2 activity. UCN-01 prevented the increase of cyclin A/cyclin-dependent kinase 2 activity induced by CPT and enhanced Cdc2 kinase activity. Replication protein A (RPA2) was hyperphosphorylated after CPT treatment, and this effect was also abrogated by UCN-01. UCN-01 potentiated the cytotoxicity of CPT and reduced by 6-fold the concentration of CPT required to kill 50% of the HT-29 cells, as determined by clonogenic assays. This effect was observed at concentrations of UCN-01 that alone were not cytotoxic and had no detectable effect on cell cycle progression. UCN-01 markedly potentiated the cytotoxicity of CPT also in HCT116/E6 and MCF-7/ADR cells defective for p53 function, whereas significantly less potentiation was observed in p53-wild-type HCT116 and MCF-7 cells. These results suggest the existence of an S-phase checkpoint that delays replication and that may extend the time available for DNA repair. Thus, pharmacological abrogation of CPT-induced S- and G2-phase checkpoints by UCN-01 may provide an effective strategy for enhancing the chemotherapeutic activity of CPT, particularly against p53-defective tumors.

INTRODUCTION

UCN-01 is presently in Phase I clinical trial. It inhibits the growth of human and murine tumor cell lines in vitro and exhibits antitumor activity in animal models (1, 2). UCN-01 is a more selective protein kinase C inhibitor than staurosporine (3, 4), and cell cycle analyses show that UCN-01 can induce G2-phase block (5–8).

UCN-01 enhances the antitumor activity of mitomycin C both in vitro and in vivo on oncogene-activated human and murine tumor cell lines (2). Recent studies indicate that UCN-01 enhances cell killing by γ-irradiation both in CA46 and HT-29 cells and synergizes with cisplatin to preferentially kill MCF-7 cells with defective p53 function. This enhancement has been related to an abrogation of the G2 checkpoint and activation of Cdc2 kinase activity (9). Bunch and Eastman (10) also reported that UCN-01 abrogates the G2 arrest induced by cisplatin and enhances cisplatin-induced cytotoxicity in Chinese hamster ovary cells.

CPT is a selective topoisomerase I inhibitor (11), and its derivatives are clinically active against human cancers, such as colon and ovarian carcinomas (12–14). Previous studies indicated that CPT-induced cytotoxicity was correlated with S and G2 abnormalities in colon carcinoma cell lines (15, 16). This is probably related to the induction of DNA damage (replication fork collisions) in replicating DNA (11). Thus, CPT can be considered as an S phase-specific DNA-damaging agent (11).

In the present study, we used human colon carcinoma p53-mutant (17) HT29 cells to investigate whether UCN-01 can affect cell cycle arrest induced by CPT (15, 16). Our data demonstrate for the first time that UCN-01 abrogates CPT-induced S-phase delay and enhances CPT cytotoxicity, suggesting the existence of a UCN-01-sensitive S-phase checkpoint in HT29 cells. These effects were analyzed further at the molecular level and in two other p53-wild-type and -mutant cell lines.

MATERIALS AND METHODS

Drugs, Chemicals, and Antibodies. UCN-01 was provided by Dr. H. Nakano (Kyowa Hakko Co., Tokyo, Japan) or the Drug Synthesis Chemistry Branch, Division of Cancer Treatment, NCI. Aliquots were stored frozen at 10 m at DMSO, and diluted further in water immediately prior to each experiment.

Anticancer Drug Screen (Developmental Therapeutics Program) and grown at 37°C in the presence of 5% CO2 in RPMI 1640 supplemented with 5% fetal bovine serum (Life Technologies, Inc.). 2 mg/ml glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. HCT116 cells transfected with control vector (HCT116) and vector containing overexpressed HPV-16 E6 (HCT116/E6) were grown in the same medium containing 0.5 mg/ml G418.

Flow Cytometry. Cell cycle assays were performed as described previously (16). Briefly, cells were harvested and fixed in 70% ethanol. Before analysis by flow cytometry, cells were washed with PBS, treated with 1 mg/ml RNase, and stained with 50 µg/ml propidium iodide for at least 30 min. DNA content was determined by FACScan flow cytometry (SOBR model, 15,000 cells per sample; Becton Dickinson Immunocytometry Systems, San Jose, CA). Shown are representative data from individual experiments that were repeated twice (Figs. 1, 3, and 9).

DNA Synthesis Assay. Cells were prelabeled with 0.005 µCi/ml of [14C]thymidine (53.6 mCi/mmol), [methy1-3H]thymidine (80.9 Ci/mmol), and [γ-32P]ATP (4500 Ci/mmol) and (17) were purchased from Pharmingen (San Diego, CA). Anti-proliferating cell nuclear antigen, anti-p21, and anti-RPA2 monoclonal antibodies were from Oncogene Science, Inc. (Cambridge, MA). Antimouse immunoglobulin and horseradish peroxidase antibody was purchased from Amersham Life Science (Arlington Heights, IL).

Cancer Cell Lines, Possibly Influenced by p53 Function

[14C]Thymidine, [methy1-3H]thymidine, and [γ-32P]ATP were purchased from New England Nuclear (Boston, MA).

Cell Culture. Human colon carcinoma HT29, HCT116, and HCT116/E6 and breast cancer MCF-7 and MCF-7/ADR cells were obtained from the NCI Anticancer Drug Screen (Developmental Therapeutics Program) and grown at 37°C in the presence of 5% CO2 in RPMI 1640 supplemented with 5% fetal bovine serum (Life Technologies, Inc.). 2 mg/ml glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin. HCT116 cells transfected with control vector (HCT116) and vector containing overexpressed HPV-16 E6 (HCT116/E6) were grown in the same medium containing 0.5 mg/ml G418.

Flow Cytometry. Cell cycle assays were performed as described previously (16). Briefly, cells were harvested and fixed in 70% ethanol. Before analysis by flow cytometry, cells were washed with PBS, treated with 1 mg/ml RNase, and stained with 50 µg/ml propidium iodide for at least 30 min. DNA content was determined by FACScan flow cytometry (SOBR model, 15,000 cells per sample; Becton Dickinson Immunocytometry Systems, San Jose, CA). Shown are representative data from individual experiments that were repeated twice (Figs. 1, 3, and 9).

DNA Synthesis Assay. Cells were prelabeled with 0.005 µCi/ml of [14C]thymidine for 48 h at 37°C. The rate of DNA synthesis was measured by 10-min pulses with 1 µCi/ml of [methy1-3H]thymidine. 1H incorporation was stopped by washing cell cultures twice in ice-cold HBSS and then scraping cells into 4 ml of ice-cold HBSS. One-ml aliquots were then precipitated with 100 µl of 100% trichloroacetic acid in triplicate. Samples were vortexed, mixed, and centrifuged for 10 min at 1200 rpm at 4°C. The precipitates were then dissolved overnight at 37°C in 0.5 ml of 0.4 N NaOH. Samples were counted by dual label liquid scintillation counting, and 1H values were normalized using 14C counts. Inhibition of DNA synthesis was calculated as the ratio of 1H:14C in the treated samples over the 1H:14C ratio in the untreated control samples.

Immunoblotting. Cells were pelleted, washed once in PBS, and lysed at 4°C as described previously (18). Protein detection was performed using a...
Reactions were stopped by adding 3X SDS-gel loading buffer (NOVEX, San Diego, CA) and boiling samples for 5 min. Samples were loaded onto 12% SDS-polyacrylamide gels (NOVEX, San Diego, CA) and electrophoresed at 125 V for 2 h. For quantitation of cyclin/Cdk activity of immunoprecipitates, gels were dried, and histone H1 phosphorylation was measured using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Shown are representative data from an individual experiment that was repeated at least twice (Fig. 6).

Clonogenic Assays. Cells were treated with CPT for 8 h. CPT was removed by rinsing the cultures in complete medium without drug, and UCN-01 was added for the next 16 h. Following drug treatments, cells were washed in fresh medium and trypsinized. Two hundred to 500 cells were seeded in triplicate in T-25 tissue culture flasks with 5 ml of medium. Colonies were grown for 2 weeks and then washed with PBS, fixed with methanol, and stained with methylene blue (0.04%). Cloning efficiencies of untreated cells were 80% for HT29, 52% for HCT116, 46% for HCT116/E6, 51% for MCF-7, and 29% for MCF-7/ADR.

RESULTS

Cell Cycle Effects of CPT in the Absence and Presence of UCN-01. We first investigated whether UCN-01 affected the cell cycle distribution of CPT-treated HT29 cells. Flow cytometry analyses (Fig. 1) showed that treatment with low CPT concentration (10 nM) for 8 h produced only minimal cell cycle perturbation (limited S- and G2-phase accumulation). After CPT removal, the cells accumulated in both the S and G2 phases of the cell cycle. Addition of UCN-01 immediately after CPT removal suppressed CPT-induced S-phase accumulation (Fig. 1). The treatment protocol outlined in Fig. 2 was used subsequently unless otherwise indicated.

Two possibilities may account for the suppression of CPT-induced S-phase accumulation by UCN-01. One is that UCN-01 blocked cell entry into S phase, and the other is that UCN-01 overcame S-phase delay induced by CPT. To discriminate between these two possibilities, cells were treated with the mitotic inhibitor nocodazole immediately after CPT treatment. The treatment protocol is shown in Fig. 2. Fig. 3 shows that CPT arrested HT29 cells in S and G2 in both the absence and presence of nocodazole, indicating an S-phase arrest in the first cell cycle following CPT treatment. When the CPT-treated cells were treated with both UCN-01 and nocodazole, a large fraction of the cells were in G2-M (56%), indicating that a significant fraction of cells was not blocked in G2 under these conditions. UCN-01 alone (100 nM) did not significantly affect cell cycle progression. These results indicate that UCN-01 suppresses the S-phase accumulation induced by CPT in HT29 cells by enabling the cells to progress through the S phase.

DNA Synthesis Inhibition and Delayed S-Phase Progression Induced by CPT and Effects of UCN-01. We next tested whether the S-phase accumulation observed after CPT treatment was due to delayed S-phase progression or to delayed release from a G2 block. In the latter case, this would be equivalent to an S-phase synchronization, and DNA synthesis measured by thymidine incorporation would be expected to be high. Fig. 4 shows that CPT treatment inhibited DNA synthesis to approximately 60% of control, whereas the fraction of cells with an S-phase DNA content was increased 2.2-fold above control. This

![Fig. 1. Abrogation of CPT-induced S and G2 arrest/delay by UCN-01 in HT29 cells.](image)

Cells were treated with 10 nM CPT for 8 h, after which cells were washed in fresh medium and treated with 100 nM UCN-01 for 16 h. Cells were harvested and analyzed by flow cytometry at various times. A, control, exponentially growing untreated cells; CPT, cells treated with 10 nM CPT for 8 h; UCN-01, cells treated with 100 nM UCN-01 for 16 h. B and C, time course after CPT removal without (B) or with UCN-01 added (C); D, quantitation of data from panels A, B, and C.

![Fig. 2. Standard treatment protocol.](image)
Fig. 3. Abrogation of CPT-induced S-phase arrest by UCN-01 in HT29 cells. Cells were treated with 10 nM CPT for 8 h. Following CPT treatment, cells were washed in fresh medium and treated with 100 nM UCN-01 with (CPT + Noc, Noc) or without (Control, CPT) 0.4 μg/ml nocodazole for 16 h. Cells were harvested and analyzed for cell cycle distribution by flow cytometry. -UCN-01, no UCN-01; + UCN-01, in the presence of UCN-01.

Fig. 4. Effects of CPT and UCN-01 on DNA synthesis and S-phase distribution in HT29 cells after drug treatment. Cells were treated as described in Fig. 2. DNA synthesis was measured by 10-min pulses, as described in "Materials and Methods," and S-phase distribution was analyzed by flow cytometry. Numbers above columns, relative ratio of DNA synthesis:S-phase fraction. Data are means (bars, SD) of three independent experiments.

Fig. 5. Western blot analyses of HT29 cells treated with CPT and/or UCN-01. Cells were treated as described in the legend to Fig. 2 and examined 16 h after CPT removal.

E/Cdk activity. This was also the case for the combination of CPT and UCN-01 (Fig. 6). These results indicate that the S-phase delay induced by CPT is not related to an inhibition of cyclin A/Cdk2 activity and that abrogation of the CPT-induced G2 and S arrest or delay by UCN-01 is associated with activation of cyclin Bl/Cdc2 activity and suppression of cyclin A/Cdk2 activity.

RPA is a mammalian single-stranded DNA binding factor essential for DNA replication, and its expression is upregulated during S phase. In the absence of CPT, RPA expression is not altered. However, UCN-01 alone did not markedly affect DNA synthesis or S-phase fraction. However, cells treated with UCN-01 after CPT treatment tended to normalize their DNA synthesis and S-phase distribution.

Molecular Changes Associated with the Cell Cycle Effects of CPT in the Absence and Presence of UCN-01. We next examined whether the cell cycle effects of CPT and UCN-01 were associated with modified expression of cyclin and Cdk proteins by Western blotting (Fig. 5). Cyclin A protein was increased by CPT treatment. UCN-01 decreased cyclin A levels and prevented the cyclin A increase induced by CPT. CPT did not affect Cdc2, Cdk2, cyclin B1, or proliferating cell nuclear antigen protein levels. p21 was not detectable in p53-mutant HT29 cells in the absence or presence of drug (data not shown).

Arrest of cells in G2 phase following DNA damage has been associated with suppression of Cdc2 kinase activity (20, 21). Consistent with these earlier observations, we found that Cdc2 kinase activity in CPT-treated cells was not elevated in spite of the presence of an increased fraction of cells in G2. UCN-01 increased the Cdc2 kinase activity 3- to 4-fold in CPT-treated cells (Fig. 6). By contrast, Cdk2 kinase activity was increased in CPT-treated cells and was decreased by UCN-01 treatment (Fig. 6). CPT and UCN-01 decreased cyclin A/Cdk2 and cyclin B1/Cdc2, while cyclin D1/Cdk2 activity remained unchanged.
for DNA replication, repair, and recombination. It is composed of three subunits of Mr 70,000, 34,000, and 11,000 (RPA1, RPA2, and RPA3, respectively). Because RPA2 is a cyclin A substrate (22) and a key element of the replication complex, we studied its modifications. Fig. 7 shows that CPT induced RPA2 phosphorylation by 8 h and that RPA2 phosphorylation continued to increase following CPT removal. UCN-01 suppressed RPA phosphorylation induced by CPT, demonstrating that abrogation of the S-phase delay by UCN-01 is associated with suppression of RPA phosphorylation, consistent with the suppression of cyclin A.

**Potentiation of CPT Cytotoxicity by UCN-01.** The treatment protocol (see Fig. 2) used to demonstrate the cell cycle effects of UCN-01 and CPT was tested in cytotoxicity assays. We hypothesized that UCN-01 might enhance CPT cytotoxicity, because cells could not arrest in S phase. Under these conditions, UCN-01 alone produced limited cytotoxicity (Fig. 8). However, UCN-01 potentiated CPT cytotoxicity up to 50-fold and reduced by 6-fold the concentration of CPT required to kill 90% of the cells, as determined by clonogenic assays (Fig. 8). This effect was observed at concentrations of UCN-01 that alone were not cytotoxic.

The above results raised the question whether such a potentiation of CPT cytotoxicity by UCN-01 might be selective for the p53-mutant HT29 cells (9). We compared the effects of UCN-01 on the cytotoxicity of CPT in human colon carcinoma HCT116 cells (p53 wild type) and in HCT116/E6 cells (p53 function disrupted by transfection with the human papillomavirus type 16 E6 gene; Ref. 23). As for HT29, 0.1 \( \mu \)M UCN-01 was not cytotoxic by itself in either cell line. However, the synergistic effect of UCN-01 plus CPT was more pronounced in the HCT116/E6 than the HCT116 cells (Table 1). We also tested two breast carcinoma cell lines, MCF-7 cells (p53 wild type), and MCF-7/ADR cells (p53 mutant; Table 1) and found that the UCN-01 potentiation was greater in the MCF-7/ADR cells. These results demonstrate that UCN-01 potentiates CPT-induced cytotoxicity in three different p53-deficient cell lines.

**S-Phase Delay Induced by CPT in Synchronized HT29 Cells and Its Abrogation by UCN-01.** To further study the CPT-induced S-phase arrest and its modulation by UCN-01, HT29 cells were synchronized at the G1-S boundary by double-aphidicolin block. One hour after removal of aphidicolin, CPT was added either alone or with UCN-01. Cells were analyzed for cell cycle distribution at various times (Fig. 9). The untreated cells were in mid-S phase by 4 h, late S phase by 7 h, and G2 phase by 10 h. CPT caused delayed S-phase progression with marked accumulation of cells in S phase at 10 h. UCN-01 by itself did not affect cell cycle progression. However, addition of UCN-01 to CPT-treated cells restored cell cycle progression as the cells were in G2 phase at 10 h (Fig. 9B). These results are consistent with an abrogation of the CPT-induced S-phase delay by UCN-01.

**DISCUSSION**

In the present study, we found that nanomolar concentrations of CPT delayed cell cycle progression through S phase and that UCN-01 abrogated this S-phase delay and potentiated CPT cytotoxicity in p53-mutant human colon carcinoma HT29 cells. These results suggest that UCN-01 is a potent abrogator of both the S and G2 checkpoints. G2 checkpoint abrogation by UCN-01 was reported recently with two other DNA-damaging agents, cisplatin and ionizing radiation (9, 10).

---

ABROGATION OF S-PHASE CHECKPOINT BY UCN-01

Table 1 Effect of UCN-01 on CPT cytotoxicity in wild-type and p53-deficient HCT116 and MCF-7 cells

<table>
<thead>
<tr>
<th>Drug Treatment</th>
<th>HCT116</th>
<th>MCF-7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Wild-type p53</td>
<td>E6 (p53 deficient)</td>
</tr>
<tr>
<td>CPT (3 nm)</td>
<td>70.4 ± 2.0 (3)</td>
<td>76.5 ± 4.7 (3)</td>
</tr>
<tr>
<td>UCN-01 (100 nm)</td>
<td>97.4 ± 0.6 (3)</td>
<td>96.2 ± 1.3 (3)</td>
</tr>
<tr>
<td>CPT + UCN-01</td>
<td>61.0 ± 1.2 (3)</td>
<td>30.1 ± 2.2 (3)</td>
</tr>
</tbody>
</table>

* Cells were treated with CPT and UCN-01 as described in Fig. 2. Cell survival was determined by clonogenic assays. Values shown are the mean ± SD. Numbers in parentheses indicate the number of independent experiments with triplicate determination.

* $P < 0.005$ compared with wild type.

To our knowledge, this is the first report suggesting pharmacological abrogation of an S-phase checkpoint by UCN-01 in response to DNA damage.

S-phase delay following DNA damage is associated with a block of replication initiation as well as suppression of DNA elongation (24, 25). Passage through $G_1$ into S phase is regulated by the activities of cyclin D-, E-, and A-associated kinases. The accumulation of cyclin E is highly periodic, peaking during late $G_1$ and declining during S phase. Thus, cyclin E is a key regulator of the $G_1$-to-S phase transition (26, 27). The activation of cyclin A/Cdk2 following that of cyclin E/Cdk2 is essential for S-phase progression (28, 29). Cyclin A binds both Cdk2 and Cdc2, giving two distinct cyclin A kinase activities, one appearing in S phase and the other in $G_2$ (29). Our data show that abrogation of CPT-induced S-phase delay by UCN-01 was associated with a marked decrease of CPT-induced cyclin A/Cdk2 activation and RPA2 phosphorylation. RPA2 is a known substrate of cyclin A/Cdk2 with an essential function in DNA replication (22). These observations indicate that CPT-induced S-phase delay is exerted logically downstream from cyclin A/Cdk2 kinase and RPA2 hyperphosphorylation. It is possible that CPT-induced delay in the S phase allows the release of control CPT UCN-01 CPT+UCN-01

Fig. 9. Effects of CPT and UCN-01 on cell cycle progression in aphidicolin-synchronized HT29 cells. Cells were synchronized in $G_1$-$S$ phase using double aphidicolin block (16-h aphidicolin treatments with a 12-h interval). Cells were released into cycle by washing in fresh medium, and 1 h later they were treated with 10 nm CPT and/or 30 nm UCN-01 for the indicated times. A, cells were analyzed by flow cytometry; B, quantitation of data in A.
accumulation of abnormally high cyclin A levels. The reduced cyclin A/Cdk2 activity associated with UCN-01 exposure may be secondary to cells having progressed out of S phase. Alternatively, activation of cyclin A/Cdk2 kinase may directly affect DNA replication, given that DNA polymerase α-prime phosphorylation by cyclin A/Cdk2 has recently been shown to inhibit the initiation of SV40 DNA replication in vitro (30).

CPT also arrests cells in G2, and we previously reported a correlation between G2 checkpoint deficiency and CPT cytotoxicity (15, 16). Entry into mitosis is regulated by the formation and activation of the cyclin B1/Cdc2 kinase complex, which requires p34cdc2 dephosphorylation (31). DNA damage-induced G2 arrest is associated with the accumulation of relatively inactive cyclin B1/Cdc2 complexes (20, 32). Our data are consistent with this, inasmuch as they show lack of cyclin B1/Cdc2 kinase activation in the G2-arrested cells after CPT treatment. Furthermore, we found that UCN-01 markedly enhanced cyclin B1/Cdc2 kinase activity following CPT treatment, which is consistent with G1-M transition and G2 checkpoint abrogation by UCN-01 (9, 10).

Our data suggest a greater potentiation of CPT cytotoxicity by UCN-01 in p53-deficient cells. A recent study suggested that p53 deficiency enhanced the potentiation of ionizing radiation- and cisplatin-induced cytotoxicity by UCN-01 (9, 10). The interpretation was that p53-deficient cells, because of their G1 checkpoint deficiency, would have to rely on the G2 checkpoint to repair DNA. For CPT, the situation might be different, because the DNA damage induced by the topoisomerase I-cleavable complexes is linked to DNA replication (33–35). Thus, it would appear that the S and G2 checkpoints might be critical in the case of CPT. How p53 might play a role in the S and G2 checkpoints is not clear. p53 has been reported to bind to damaged DNA and facilitate DNA repair (36, 37). p53-deficient cells have also been reported to exhibit G2 checkpoint deficiency (38–40). Thus, it is possible that UCN-01 might more easily overcome deficient cell cycle checkpoints. DNA repair could aid the survival of damaged cells progressing through S and M. When UCN-01 impairs S and G2 checkpoints in CPT-treated cells, impairment of p53-dependent DNA repair could thus further reduce cell survival. From a therapeutic standpoint, the greater activity of the combination CPT/UCN-01 might provide a rationale for selectivity against p53-mutant tumors.

In summary, our studies suggest the existence of an S-phase checkpoint that delays replication while DNA damage is present. Such a checkpoint would be abrogated by UCN-01. Cell cycle checkpoint deficiencies may contribute to the selectivity of CPT for cancer cells (16). Modulation of cell cycle checkpoints may provide new strategies to selectively enhance the cytotoxicity of DNA-damaging drugs.

REFERENCES


ABROGATION OF S-PHASE CHECKPOINT BY UCN-01


Abrogation of an S-Phase Checkpoint and Potentiation of Camptothecin Cytotoxicity by 7-Hydroxystaurosporine (UCN-01) in Human Cancer Cell Lines, Possibly Influenced by p53 Function

Rong-Guang Shao, Chun-Xia Cao, Tsunehiro Shimizu, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/57/18/4029

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.
Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.