7-Hydroxy staurosporine (UCN-01) Causes Redistribution of Proliferating Cell Nuclear Antigen and Abrogates Cisplatin-induced S-Phase Arrest in Chinese Hamster Ovary Cells

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Abstract

A variety of agents, such as caffeine, have been shown to abrogate the DNA damage-dependent G2 checkpoint and enhance cytotoxicity. However, these agents are too toxic for clinical use. We have reported that the potent protein kinase inhibitor 7-hydroxy staurosporine (UCN-01) at nontoxic doses abrogates the G2 arrest caused by the DNA-damaging agent cisplatin. Here, using Chinese hamster ovary cells, we show that cisplatin causes predominantly an S-phase arrest; UCN-01 abrogates this S-phase arrest, causing progression of cells to G2 and, subsequently, apoptotic cell death. In searching for an explanation for this accelerated DNA synthesis, we discovered that UCN-01 causes translocation of proliferating cell nuclear antigen (PCNA) to the detergent-insoluble, DNA-bound fraction. PCNA acts as a sliding clamp for DNA polymerase δ. Sequestration of PCNA by p21waft/cdpl is required for p53-dependent G2 arrest in damaged cells. However, the S-phase arrest occurs independently of p53 and p21waft/cdpl. Our results suggest that PCNA is also a component of this S-phase checkpoint, despite the fact that CHO cells are defective for p53, and no increase in p21waft/cdpl was observed. The mechanism by which PCNA is sequestered in the absence of p21waft/cdpl and the mechanism by which UCN-01 disrupts this sequestration remain to be elucidated.

Introduction

Progression through the cell cycle is regulated carefully at various checkpoints to avoid proliferation when adverse conditions exist. For example, cells with damaged DNA arrest at checkpoints in G1 or S phase to prevent replication on damaged DNA or in G2 to prevent aberrant mitosis (1, 2). The function of these checkpoints is to provide more time to repair damaged DNA prior to replication or mitosis, thereby enhancing cell survival and reducing the probability of mutation and carcinogenesis. The p53 tumor suppressor protein is required for the DNA damage-inducible G2 arrest (3). In a normal cell, DNA breaks cause an increase in the p53 protein, which in turn induces transcription of p21waft/cdpl, an inhibitor of cdkss (4) required for cell cycle progression (4). In contrast, cells defective in p53 still arrest in either the S or G2 phase, showing that p53 is dispensable for these checkpoints.

The DNA damage-inducible G2 arrest has been best studied in the yeast Saccharomyces cerevisiae. A RAD9 mutant phenotype has been identified in which cells are hypersensitive to DNA-damaged agents because they undergo a lethal mitosis rather than arresting in G2 (5). At least six genes have been identified in the RAD9 phenotype; of these, two are also required for S-phase arrest (6). Only one of these six genes has thus far been shown to have homology to a human gene. The yeast mecl gene, which is required for both S and G2 arrest, is homologous to the human ATM gene, the deficiency of which is responsible for ataxia telangiectasia; this disease is characterized by hypersensitivity to DNA damaging agents and an inability to arrest DNA synthesis (7, 8). The ATM gene is also involved in the DNA damage-dependent increase in p53, so it is also likely to play a role in the G1 checkpoint (9).

The enhanced sensitivity of RAD9 mutants to DNA-damaging agents suggests a mechanism to enhance the activity of many anticancer drugs. Indeed, pharmacological agents that abrogate the G2 checkpoint and enhance cytotoxicity have been known for many years (10, 11). The prototype drug in this regard is caffeine. Recently, it was discovered that caffeine and related methylxanthines abrogate the G2 checkpoint preferentially in the absence of p53 (12–14). Hence, although p53 is not required to activate this checkpoint, it does inhibit the abrogation of the checkpoint. Accordingly, the addition of caffeine following a DNA-damaging agent may preferentially enhance killing in p53-defective tumors while sparing the normal host tissue. The problem with this potential therapeutic strategy is that patients cannot tolerate the doses of caffeine required. In searching for better drugs

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3 The abbreviations used are: cdk, cyclin-dependent kinase; CHO, Chinese hamster ovary; PCNA, proliferating cell nuclear antigen; UCN-01, 7-hydroxy staurosporine; TBS, Tris-buffered saline.
that abrogate the G2 checkpoint, we discovered that UCN-01 is 100,000 times more potent than caffeine (15). UCN-01 abrogates the G2 checkpoint at nontoxic concentrations, and these concentrations are reported to be well tolerated in animal studies (16). This drug dramatically sensitizes cells to the DNA-damaging drug cisplatin (15) and also functions preferentially when p53 is defective (17). Accordingly, we are optimistic that this combination will have a significant therapeutic activity.

CHO cells are functionally defective for p53, although the specific mutation is unknown (18). In our initial studies with UCN-01, we used CHO/UV41 cells, selected because of their deficiency in DNA repair (15, 19). Accordingly, once incubated with cisplatin, the level of cisplatin-induced DNA damage does not vary during subsequent incubations. In this model, low concentrations of cisplatin produce no detectable slowing of passage through S phase but a marked G2 arrest. Although UCN-01 abrogates the G2 checkpoint within 4 h, it does not enhance cisplatin cytotoxicity, as the lack of DNA repair already makes the cells hypersensitive. In contrast, incubation of DNA repair-competent CHO/AA8 cells with UCN-01 reduces by 3-fold the amount of cisplatin required to kill 90% of the cells (15). This effect occurs at higher concentrations of cisplatin that cause a significant accumulation of cells in the G2 phase. Here, we investigate the effect of UCN-01 on this G2-phase checkpoint. We show that UCN-01, like early reports with caffeine (20), enhances the rate of DNA synthesis on a damaged DNA template. We show that this effect is not mediated through activation of cdk1, a previously suggested target for both caffeine and UCN-01 during abrogation of the G2 checkpoint (19, 21–24). Finally, we show that UCN-01 mediates redistribution of PCNA to the replication complex thereby facilitating DNA synthesis. These results suggest a mechanism for the regulation of the DNA damage-induced G2-phase cell cycle checkpoint.

Results

UCN-01 Abrogates Cisplatin-induced S-Phase Arrest.

Our previous experiments on the effects of UCN-01 involved analysis of cell cycle progression in a DNA repair-deficient cell line, CHO/UV41 (15). Incubation with 0.5 μg/ml cisplatin induced almost exclusively a G2 arrest, which was abrogated rapidly by incubation with 50 nM UCN-01. Most cell lines are DNA repair competent and therefore require higher concentrations of cisplatin to alter cell cycle progression. For example, incubation of CHO/AA8 cells with 0.5 μg/ml cisplatin causes little accumulation of cells in any phase of the cell cycle, whereas 5 μg/ml cisplatin causes a marked accumulation of cells in S phase (25). In the present experiments, we have investigated the ability of UCN-01 to abrogate this S-phase arrest in CHO/AA8 cells after cisplatin treatment.

Cells were treated with 5 μg/ml cisplatin for 2 h and then incubated for various times in the presence or absence of 50 nM UCN-01 (Fig. 1). Incubation of cells with UCN-01 alone for 24 h did not alter the cell cycle distribution compared to control asynchronous cells; this is consistent with the lack of any cytotoxicity at this concentration of UCN-01 (15). Cells incubated with cisplatin for 2 h showed a reduction in the number of G1 cells 6 h later, indicating that cisplatin had decreased the number of cells completing mitosis. By 13 h, the majority of cells appeared in S phase, and these progressed slowly to late S and G2 by 24 h; this is contrasted to a 6–8-h S phase in undamaged cells (19). Thus, cisplatin treatment greatly reduced the rate of S-phase progression.

The effect of UCN-01 on this S-phase delay was examined by adding 50 nM UCN-01 to cells immediately after cisplatin. By 13 h, many cells had already progressed to late S and G2 phase, and with continued incubation, many cells began to appear with sub-G1 DNA content indicative of dying cells (Fig. 1). This increased cell cycle progression can be seen even more clearly when UCN-01 was added to cells that had already arrested in S phase 13 h after cisplatin treatment. The cells progressed rapidly through S phase into G2, and many cells subsequently appeared to die as judged by the cells with sub-G1 DNA content.

Cells with sub-G1 DNA content are an indication of apoptotic cell death; such cells have fragmented their DNA, and this is lost during fixing in ethanol. However, quantitation of this sub-G1 population is invalid, because cells frequently fragment into multiple apoptotic bodies. To more directly assess apoptosis, cells were harvested and analyzed by gel electrophoresis for DNA fragmentation. In cells incubated only with cisplatin, no significant DNA fragmentation was observed during the subsequent 24 h (Fig. 2). Similarly, incubation with UCN-01 alone induced no DNA digestion. In contrast, the addition of UCN-01 13 h after cisplatin resulted in a distinct nucleosome ladder by 24 h.

An alternate explanation for the changes in cell cycle distribution following addition of UCN-01 is preferential death of cells in early S phase, which would give the appearance of more cells in late S and G2 phase. To confirm that UCN-01 was indeed accelerating the rate of DNA synthesis, we measured the incorporation of [3H]thymidine. Cells were incubated with cisplatin for 2 h, and various concentrations of UCN-01 were added after an additional 13 h, as in Fig. 1. At the time of addition of UCN-01, [3H]thymidine was also added. Cells were harvested and analyzed for incorporation of radioactivity over the following 3 h (Fig. 3). Incubation with 10 μg/ml cisplatin suppressed DNA synthesis more dramatically than 5 μg/ml. The addition of 10 nM UCN-01 had minimal effect, whereas 50 nM was almost as effective as 200 nM at accelerating DNA synthesis. Hence, the concentrations of UCN-01 required to abrogate S-phase arrest are similar to those previously found effective at abrogating G2 arrest (15).

These results indicate that cells respond to cisplatin treatment by inhibition of DNA synthesis and slowed progression through both S and G2 phases. The rate of progression through each of these phases of the cell cycle is considered to be regulated by checkpoints designed to facilitate repair of potentially lethal damage. UCN-01 overcomes the S-phase checkpoint and enhances cell death. Previously, we demonstrated a much more pronounced G2 arrest in the DNA repair-deficient cell line UV41 (using lower concentrations of cisplatin), but this checkpoint was abrogated at the same concentration of UCN-01 (15). However, in those experiments, a significant G1 population occurred prior to cell death. In the current model, no G1 population appeared,
possibly because of extensive chromosome damage resulting from forced replication on the damaged DNA template.

UCN-01 Does Not Prematurely Induce cdk1 Dephosphorylation. Abrogation of the DNA damage-dependent G\(_2\) checkpoint has previously been associated with activation of the cyclin dependent kinase cdk1 (also known as p34\(^{\text{cdc2}}\)) (21–24). We have previously shown activation of cdk1 when cisplatin-damaged UV41 cells were incubated with caffeine and thereby forced to undergo mitosis (19). Experiments with UCN-01 as a single agent have also shown activation of cdk1 and a shortened G\(_2\) phase (24). These results all suggest that cdk1 may be the target, or closely related to the target, for these mitotic-inducing agents. In the current model, we have observed abrogation of an S-phase checkpoint and therefore have questioned whether cdk1 might also play some role in abrogating this checkpoint.

CHO cells were incubated with cisplatin and UCN-01 according to the same protocol described above. Cells were harvested at various times after cisplatin treatment and/or UCN-01 treatment and immediately lysed, electrophoresed, and probed by Western blotting for cdk1 phosphoforms. The character of these phosphoforms has been studied extensively (26–28). The newly synthesized unphosphorylated cdk1 migrates faster during electrophoresis. An initial, activating phosphorylation (threonine 161) does not alter the migration. The inactivating phosphorylations (threonine 14 and tyrosine 15) lead to a more slowly migrating form. Activation of cdk1 at mitosis results from removal of the inactivating phosphates and return to the faster migrating form.

Asynchronous control CHO cells show predominantly the faster migrating form of cdk1 (Fig. 4, A and B). Following incubation of the cells with cisplatin, a marked increase is observed in the hyperphosphorylated form of cdk1 at 13 h, and this increase is retained throughout the 21 h of the experiment. UCN-01 was added at 13 h and caused minimal change in the phosphoforms compared to the same time points in the absence of UCN-01 (a small degree of dephosphorylation may occur, as a few cells are in G\(_2\)). However, at 21 h, UCN-01 caused a marked dephosphorylation of cdk1. These results show that activation of cdk1 is not associated with UCN-01-mediated abrogation of the S-phase delay; rather, cdk1 appears to be activated only after the cells reach the G\(_2\) phase, the point in the cell cycle at which activation normally occurs.

To determine whether it is necessary to complete DNA synthesis prior to dephosphorylation of cdk1, cells were
through gel from cisplatin and during mentalization arrest cells. Preceding abrogation of the S-phase checkpoint, these cells were incubated concurrently with UCN-01 and aphidicolin to inhibit the DNA polymerase. When aphidicolin was added 13 h after cisplatin, it prevented the UCN-01-mediated progression of cells through the cell cycle (Fig. 4C). Aphidicolin also prevented the dephosphorylation of cdk1 that was otherwise induced by UCN-01 at 21 h (Fig. 4B). These results confirm that cells must reach G2 prior to activating cdk1, and hence that UCN-01 is not abrogating the S-phase checkpoint through any action directed toward cdk1.

**UCN-01 Induces a Redistribution of PCNA.** The above results indicate that UCN-01 rapidly induces DNA synthesis and abrogation of the S-phase arrest in cisplatin-damaged cells. This suggests that UCN-01 may target the replication machinery. PCNA is a component of the replication complex that is required for S-phase progression. During p53-dependent G2 arrest, PCNA is sequestered in a complex with p21\textsuperscript{wt1/cop1} (29). The distribution of PCNA during S-phase arrest has not been established; nor has the influence of p53 status on this distribution. CHO cells are defective for p53 (18). Furthermore, we investigated the expression of p21\textsuperscript{wt1/cop1} following incubation of CHO cells with cisplatin and have observed neither constitutive nor induced protein (data not shown). We therefore investigated the compartmentalization of PCNA and the potential effect of UCN-01 during its abrogation of the S-phase checkpoint.

The total level of PCNA remains constant in logarithmically growing cells but exists in two compartments distinguishable by their differential solubility. For example, a fraction of PCNA in S-phase cells is insoluble in detergent, whereas cells at other phases of the cell cycle show no insoluble PCNA (30–32). The insoluble PCNA is thought to represent the functional form bound to DNA at the replication fork. Thus, we analyzed detergent-soluble and insoluble PCNA using the above conditions. Analysis of total cell lysates showed constant amounts of PCNA throughout the experiment (Fig. 5A). In asynchronous control cells, the majority of PCNA was present in the detergent-soluble fraction. Following cisplatin treatment, there was a slight increase in the detergent-insoluble PCNA, probably due to the larger number of cells in S phase. However, when UCN-01 was added to cisplatin-arrested cells for 2, 4 or 6 h (samples harvested at 15, 17, or 19 h, respectively), there was a marked increase in insoluble PCNA. Even under these conditions, only a slight reduction was observed in the soluble PCNA (note that to enhance detection, the immunoblot for insoluble PCNA was exposed to film for approximately 5-fold longer time periods than the immunoblots for total and soluble PCNA). This latter observation is consistent with the report that PCNA is present in considerable excess over the amount involved directly in DNA replication (33). We conclude that the UCN-01-mediated increase in insoluble PCNA results from a redistribution of a small percentage of total PCNA into the replication complex, and that this facilitates the increased DNA synthesis.

A second experiment was performed in which UCN-01 was added for 2-h periods at various times after cisplatin (Fig. 5B). PCNA was again seen to increase in the insoluble fraction. Even after 23 h when most of the cells have already reached G2 in the absence of UCN-01, the addition of UCN-01 caused an increase in PCNA in the insoluble fraction. This raises the possibility that the G2 arrest that occurs in damaged cells is more accurately a late S-phase arrest, and that UCN-01 also abrogates this arrest by causing redistribution of PCNA (see below).

The redistribution of PCNA to the replication complex could be an indirect consequence of UCN-01-mediated action; that is, UCN-01 might enhance the rate of DNA synthesis by some other mechanism, and PCNA translocates to the replication complex in response. Alternately, UCN-01 may act directly on PCNA regulation and mediate its translocation to the replication complex. To distinguish these possibilities, cisplatin-arrested cells were incubated simultaneously with UCN-01 and aphidicolin, the latter agent to prevent S-phase progression, as shown in Fig. 4C. Under these conditions, PCNA still increased in the insoluble fraction (Fig. 5C), suggesting that ongoing proliferation was not required for this redistribution. Incubation of cells with cycloheximide to inhibit protein synthesis, conditions that also prevented progression of S-phase-arrested cells (data not shown), also did not prevent the redistribution of PCNA. These results suggest that UCN-01 functions at a posttranslational step in the pathway regulating PCNA compartmentalization.

As a control for the above experiments, we also investigated the distribution of PCNA in the absence of cisplatin-
induced DNA damage. Incubation of asynchronous cells with 50 nM UCN-01 for 4 h did not increase the level of detergent-insoluble PCNA (Fig. 5D). Incubation of 50 nM UCN-01 for 13 h caused an increase in the amount of insoluble PCNA consistent with an increased number of cells in S phase due to arrest of the DNA polymerase. Interestingly, the addition of UCN-01 to aphidicolin-arrested cells caused a further increase in insoluble PCNA, confirming further that ongoing replication is not required for this redistribution. Finally, cells incubated with 0.4 μg/ml nocodazole, which causes a mitotic arrest, showed only low levels of insoluble PCNA, which was not increased by addition of UCN-01. Under none of these conditions was there any increase in total PCNA (data not shown). Hence, the ability of UCN-01 to increase the amount of insoluble PCNA occurs only in S phase-arrested cells. This arrest can be mediated either by DNA damage, such as that induced by cisplatin, or by direct block to the DNA polymerase with aphidicolin. We conclude that UCN-01 is directly or indirectly modifying the mechanism of PCNA compartmentalization.

Mechanism of G2 Arrest in CHO/UV41 Cells. The results presented above relate to the regulation of the S-phase checkpoint, but they raise the possibility that similar mechanisms may be involved in the G2 checkpoint. It is possible that the apparent G2 arrest observed during flow cytometry more correctly reflects cells arrested at late S phase. If true, then abrogation of the G2 arrest may be prevented by aphidicolin. To test this possibility, we used the CHO/UV41 cells, in which a highly synchronized G2 arrest can be obtained. The methodology and controls have been reported previously (19). Briefly, cells are synchronized by mitotic shake followed by incubation in aphidicolin. These cells are treated with cisplatin for 2 h and then incubated for 10 h, at which time they are all arrested at G2. The addition of 50 nM UCN-01 at 10 h caused complete abrogation of this G2 arrest (Fig. 6). Addition of aphidicolin concurrently with UCN-01 did not prevent abrogation of the G2 arrest. To further confirm this observation, we incubated cells with aphidicolin for 2 h prior to addition of UCN-01; again, aphidicolin did not prevent abrogation of the G2 arrest. Considering that aphidicolin prevents progression of PCNA-dependent polymerase δ, these results suggest that G2 arrest differs from S-phase arrest in that it involves a PCNA-independent mechanism.

Discussion
The focus of much recent research has been on understanding the DNA damage-dependent G2 checkpoint as a result of a growing understanding of the role of cdk complexes in the regulation of mitosis. However, identification of the S-phase arrest by DNA-damaging agents and bypass by caffeine actually predates analysis of the G2 arrest (20, 34). At that time, the model for S-phase arrest was that the DNA polymerase was blocked directly at DNA lesions, and caffeine inhibited sealing of gaps in the daughter strand opposite the offending lesions (20). However, this S-phase arrest is now considered to be a checkpoint that prevents initiation of replication on damaged DNA. Specific proteins that regulate this checkpoint have been identified in yeast (5, 6) and humans, the latter associated with the disease ataxia telangiectasia (7, 8). In the current experiments, we have investigated the S-phase arrest caused by cisplatin and the ability of UCN-01 to overcome this arrest.

Our previous work involved analysis of the G2 arrest induced by cisplatin in DNA repair-deficient CHO/UV41 cells (15, 19). Both caffeine and UCN-01 are effective at abrogating this arrest, although UCN-01 is 100,000 times more potent in this regard. To investigate the possibility that UCN-01, like caffeine, would enhance the cytotoxic killing induced by cisplatin, it was necessary to use DNA repair-competent cells. We showed that UCN-01 caused a dramatic enhancement of cisplatin-induced cell killing in the CHO/AAt8 cells (15). The current analysis of cell cycle perturbations in these cells showed that cisplatin caused predominantly an S-phase arrest, and that the addition of UCN-01 caused acceleration of DNA synthesis, progression to G2, and subsequent apoptotic cell death.

It has recently been shown that UCN-01 activates cdk1, leading to a rapid onset of mitosis. This has led to the suggestion that cdk1, or a closely related signaling component, is the target for UCN-01 (17, 24). Considering that cdk1 is a kinase required specifically for mitosis, we thought it unlikely that it would contribute to S-phase arrest or bypass. Indeed, incubation of S phase-arrested cells with UCN-01 did not activate cdk1 until the cells had progressed through S to G2, a time when the cells make an abortive attempt to undergo mitosis, the consequence of which is apoptotic cell death.
Fig. 4. UCN-01 does not cause dephosphorylation of cdk1 in S phase-arrested cells. A, cells were incubated with 5 μg/ml cisplatin for 2 h, and harvested at the indicated times. In the right four lanes, cells were incubated with 50 μM UCN-01 from 13 h until harvest. Lane I, untreated cells. Cells were lysed and analyzed by Western blots for cdk1 phosphorylation status. B, at 13 h after cisplatin, 4 μg/ml aphidicolin, 50 μM UCN-01, or the combination of aphidicolin and UCN-01 was added until harvest at 21 h. C, cells in B were harvested at the indicated times and analyzed for DNA content by flow cytometry. –, no addition of drugs.

deployment. Accordingly, cdk1 is not the target for UCN-01-mediated abrogation of the S-phase checkpoint, in contrast to its suggested role in the G2 checkpoint. This obviously raises the question as to whether cdk1 is a target for UCN-01 at the G2 checkpoint or whether its activation is just a consequence of the cell being at the appropriate phase of the cell cycle. We tested the possibility that the observed G2 checkpoint occurs at late S phase while the cell awaits completion of replication on damaged DNA. However, aphidicolin did not prevent G2 bypass, suggesting that such cells have completed DNA synthesis, at least synthesis dependent on aphidicolin-sensitive DNA polymerase α, β, or ε. We believe the target for UCN-01 is probably at a common upstream step that regulates arrest at both S and G2 phases. UCN-01 was developed as a relatively specific inhibitor of protein kinase C (35), and it remains possible that abrogation of the cell cycle checkpoints is a consequence of interaction with this target.

DNA damage also activates a DNA damage-dependent G1 checkpoint, which prevents cells from entering S phase. The current paradigm suggests that sequestering of PCNA contributes to this G1 arrest by preventing DNA synthesis. PCNA forms a trimeric structure that is tightly bound around the DNA helix, and this is thought to stabilize the DNA polymerase on the DNA (36). Hence, when PCNA is sequestered in cyclin/cdk/p21 complexes, DNA synthesis cannot occur. The G1 checkpoint is only activated in cells that express the wild-type p53 protein; this leads to an increase in p21, which is involved directly in sequestering PCNA. S-phase arrest still occurs in the absence of p53 and p21, as in the CHO cells used here. Considering the role of p21 in regulating PCNA compartmentalization, we investigated the distribution of PCNA during S-phase arrest in p53-defective CHO cells. We thought originally that PCNA might stay associated with DNA in these cells because of the absence of p21. However, our
Fig. 5. UCN-01 induces redistribution of PCNA in CHO cells arrested in S phase by cisplatin. A, cells were incubated with 5 μg/ml cisplatin for 2 h, washed, and incubated until harvest at the indicated times. Alternately, 50 nM UCN-01 was added at 13 h until harvest. At harvest, cells were lysed to obtain total PCNA or extracted with detergent to separate soluble from insoluble PCNA. Proteins were separated by electrophoresis, Western blotted, and probed with an anti-PCNA antibody. B, cells were incubated as in A, except that UCN-01 was added 2 h before harvest. Only the detergent-insoluble PCNA is shown.

C, cells were incubated as in A, except that 4 μg/ml aphidicolin or 10 μg/ml cycloheximide were added at 13 h until harvest. D, cells were incubated with 4 μg/ml aphidicolin or 0.4 μg/ml nocodazole for 16 h, with 50 nM UCN-01 added from 13–16 h, as indicated. –, no addition of drug.

results suggest that PCNA still dissociates from the DNA during S-phase arrest. The subsequent addition of UCN-01 to these S phase-arrested cells caused a marked relocation of PCNA to the insoluble, DNA-bound fraction, confirming that PCNA does dissociate from the DNA during S-phase arrest. In cells expressing wild-type p53, it is feasible that p21 could be involved in the S-phase checkpoint by sequestering PCNA. However, this is not possible in p53-defective cells in which p21 is not induced. This raises the interesting question of what p53- and p21-independent mechanism is
required for the compartmentalization of PCNA at the S-phase checkpoint.

Although p53 is not required for S or G2 arrest, it does appear to make cells resistant to the action of caffeine and UCN-01; i.e., abrogation of the DNA damage-dependent G2 checkpoint occurs preferentially in cells defective in p53 (17). It is possible that abrogation of the S-phase checkpoint may also occur preferentially when p53 is defective. Hence, the potential difference between cells with wild-type or mutant p53 with respect to abrogation of the S-phase checkpoint and compartmentalization of PCNA will be important for understanding the molecular mechanism of action of UCN-01.

The results obtained with cells incubated with aphidicolin need comment. Cells incubated only with aphidicolin for 16 h showed a slight increase in insoluble PCNA consistent with the accumulation of cells in S phase. However, addition of UCN-01 caused a further translocation of PCNA to the DNA, although the replication complex cannot progress in the continued presence of aphidicolin. According to the current model for initiation of DNA synthesis, primase and DNA polymerase α synthesize short stretches of RNA and DNA, to which PCNA is recruited, and then DNA synthesis is switched to polymerase δ and possibly ε (37). As aphidicolin inhibits DNA polymerase α, it should prevent the DNA extension that occurs prior to recruitment of PCNA. However, in the presence of aphidicolin, UCN-01 still caused PCNA to translocate to the DNA, suggesting that DNA polymerase α is not essential prior to this step. Hence, UCN-01 may mediate the recruitment of PCNA to the RNA primer. This raises the question of the state of DNA replication when cells arrest after cisplatin. It is possible that cells continually prime DNA synthesis, but the lack of available PCNA prevents elongation. Alternatively, S-phase arrest may result from inhibition of the primase step of DNA synthesis; if this is the case, then the translocation of PCNA to DNA may only indicate successful priming of DNA synthesis and not be a direct regulator of replication.

In summary, these experiments have demonstrated the effectiveness of UCN-01 at abrogating the S-phase arrest caused by cisplatin. UCN-01 overcomes the suppression of DNA synthesis such that the cells progress to G2 and subsequently undergo apoptotic cell death. We have shown that UCN-01 causes PCNA to translocate to the insoluble, DNA-bound fraction consistent with its role in facilitating DNA synthesis. Sequestering of PCNA by cyclin/cdk/p21 com-

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Fig. 6. Abrogation of G2 arrest in CHO/UV41 cells is not inhibited by aphidicolin. CHO/UV41 cells were synchronized at G1-S, incubated with 0.5 μg/ml cisplatin for 2 h, washed, and incubated until harvest at the indicated times. Aphidicolin (4 μg/ml) was added to the indicated samples at 10 h, and 50 μM UCN-01 was added at either 10 or 12 h. At harvest, cells were fixed and analyzed by flow cytometry for DNA content.
plexes has previously been implicated in the DNA damage-dependent G$_2$ checkpoint, and we suggest that a similar compartmentalization of PCNA may prevent abrogation of S-phase checkpoint. The concentration of UCN-01 required for S-phase progression is the same as required for G$_2$ bypass; this concentration of UCN-01 is nontoxic but dramatically enhances the cytotoxicity of cisplatin (15). When incubated with cisplatin, most cells show a more prominent S-phase arrest than a G$_2$ arrest. Accordingly, it is possible that abrogation of the S-phase arrest by UCN-01 is more important for its potential therapeutic action than its ability to abrogate the G$_2$ checkpoint.

Materials and Methods

Materials. Cell culture reagents, including α-MEM, sodium bicarbonate, horse serum, fetal bovine serum, trypsin, penicillin, and streptomycin were obtained from Life Technologies, Inc. (Grand Island, NY). All of the tissue culture plastics were obtained from Corning-Costar Corp. (Cambridge, MA). UCN-01 was provided by Dr. Edward Sausville (National Cancer Institute, NIH, Bethesda, MD). Cisplatin was obtained from Bristol-Myers Squibb (Princeton, NJ). Cycloheximide, aphidicolin, and nocodazole were obtained from Sigma Chemical Co. (St. Louis, MO) and were dissolved in DMSO (aphidicolin and nocodazole) or PBS (cycloheximide).

Cell Culture. CHO/AAB and CHO/U41 cells (obtained from American Type Culture Collection) were maintained as exponentially growing monolayer cultures in α-MEM supplemented with 2.5% fetal bovine serum, 2.5% horse serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a humidified incubator at 37°C with 5% CO$_2$.

Cell Cycle Analysis. Cell cycle analysis was performed according to a previously described procedure (19). Briefly, cells were harvested and fixed in 70% ethanol. DNA was then stained by incubating cells in PBS containing propidium iodide (100 μg/ml) and RNase A (1 mg/ml) for 30 min at 37°C. DNA content was determined on a Becton Dickinson FACScan flow cytometer. The cell cycle distribution was analyzed using the Cell Fit or Cell Quest software.

Thymidine Incorporation. Cells (3 × 10$^5$ in 2 ml) were plated in six-well plates overnight, unattached cells were removed, and 1 μCi of [methyl-3H]thymidine was added. After 0–3 h, the medium was removed, and the cells were rinsed in PBS, then trypsinized, collected in a glass tube, and rinsed again in PBS. An equal volume (1 ml) of ice-cold 10% trichloro-acetic acid was added, and the precipitate was allowed to form on ice for 10 min. The precipitate was collected on a 25-mm GF/C Whatman filter and washed with 5% trichloroacetic acid and ethanol prior to scintillation counting.

DNA Fragmentation. One million cells were added directly to the wells of a 2% agarose gel, where they were lysed and digested with RNase A. The DNA was electrophoresed into the gel and stained with ethidium bromide. In this method, high molecular weight DNA remains in or near the well, while nucleosome-length fragments are resolved in the gel (38, 39).

Analysis of cdk1 Phosphoforms and p21$^{waf1/cip1}$. Cells were analyzed for cdk1 essentially as described previously but with some modifications (19). Cells were harvested by scraping and washed with PBS containing 1 ml NaVO$_3$. Cells were then resuspended in lysis buffer [1% SDS, 100 mM NaCl, 20 mM Tris (pH 7.8), 5 mM EDTA, 2 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A] containing phosphatase inhibitors 10 μg/ml NaPP$_1$, 1 ml NaVO$_3$, and 1 ml NaF. For each sample, 10 μg of protein were electrophoresed on a 12% polyacrylamide/SDS gel followed by transfer to polyvinyldiene fluoro membranes (Immobilon-P; Millipore, Marlborough, MA). Membranes were blocked in 5% milk and TBS-0.05% Tween 20, incubated with a polyclonal anti-cdk1 antibody (Life Technologies, Inc.) and a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA), and washed with TBS-0.05% Tween 20 and TBS before visualization with enhanced chemiluminescence and Hyperfilm (Amer sham Corp., Arlington Heights, IL).

Analysis of p21$^{waf1/cip1}$ was similar to that for cdk1 except that protein equivalent to 10$^6$ cells was electrophoresed on a 10% polyacrylamide/SDS gel, and after transfer to a membrane, the protein was probed with the C-19 anti-p21 polyclonal antibody (Santa Cruz Biotechnology). PCNA. PCNA exists in two separable compartments, one soluble and the other insoluble in detergent, the latter being bound to DNA (30, 31). PCNA localization was determined based on this differential detergent solubilization. Following various drug treatments, 5 × 10$^6$ cells were lysed in 0.5 ml of 0.1% Triton X-100 in PBS for 10 min on ice. The insoluble fraction was collected by centrifugation, and the supernatant was collected as the detergent soluble fraction. The detergent-insoluble pellet was then solubilized in 0.50 ml of lysis buffer as above. Total PCNA was also assessed by lysing cells directly into lysis buffer. Lysates were then electrophoresed on a 10% polyacrylamide/SDS gel. Proteins were transferred to a membrane and probed with a monoclonal anti-PCNA antibody (Signet Laboratories, Inc.), followed by a goat antimouse horseradish peroxidase-conjugated secondary antibody.

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References


