The Chk1 Protein Kinase and the Cdc25C Regulatory Pathways Are Targets of the Anticancer Agent UCN-01*

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A checkpoint operating in the G2 phase of the cell cycle prevents entry into mitosis in the presence of DNA damage. UCN-01, a protein kinase inhibitor currently undergoing clinical trials for cancer treatment, abrogates G2 checkpoint function and sensitizes p53-defective cancer cells to DNA-damaging agents. In most species, the G2 checkpoint prevents the Cdc25 phosphatase from removing inhibitory phosphate groups from the mitosis-promoting kinase Cdc2. This is accomplished by maintaining Cdc25 in a phosphorylated form that binds 14-3-3 proteins. The checkpoint kinases, Chk1 and Cds1, are proposed to regulate the interactions between human Cdc25C and 14-3-3 proteins by phosphorylating Cdc25C on serine 216. 14-3-3 proteins, in turn, function to keep Cdc25C out of the nucleus. Here we report that UCN-01 caused loss of both serine 216 phosphorylation and 14-3-3 binding to Cdc25C in DNA-damaged cells. In addition, UCN-01 potently inhibited the ability of Chk1 to phosphorylate Cdc25C in vitro. In contrast, Cds1 was refractory to inhibition by UCN-01 in vitro, and Cds1 was still phosphorylated in irradiated cells treated with UCN-01. Thus, neither Cds1 nor kinases upstream of Cds1, such as ataxia telangiectasia-mutated, are targets of UCN-01 action in vivo. Taken together our results identify the Chk1 kinase and the Cdc25C pathway as potential targets of G2 checkpoint abrogation by UCN-01.

UCN-01 is a protein kinase inhibitor currently undergoing clinical trials for cancer treatment. UCN-01 potentiates the cytotoxicity of a variety of anticancer agents including cisplatin, camptothecin, and ionizing radiation (1–3). These latter findings have encouraged the investigation of UCN-01 in combination strageties for cancer treatment. A potential mechanism underlying the sensitization of cancer cells to DNA-damaging agents is the abrogation of cell cycle checkpoint function in the G2 phase of the cell cycle (1, 2). Interestingly, abrogation of the G2 checkpoint appears to occur preferentially in cancer cells with defective p53 tumor suppressor function (1). UCN-01-induced G2 checkpoint abrogation has previously been shown to occur through a Cdc2-dependent pathway resulting in premature activation of this mitosis-promoting kinase in DNA-damaged cells (1, 4). The molecular mechanism(s) underlying Cdc2 activation and G2 checkpoint abrogation by UCN-01 are not known.

Cdc2 is subject to multiple levels of regulation including periodic association with the B-type cyclins, reversible phosphorylation, and intracellular compartmentalization (for reviews see Refs. 5–7). Phosphorylation of human Cdc2 occurs on three regulatory sites as follows: threonine 14, tyrosine 15, and threonine 161 (8–11). Cdc2 is retained in an inactive state throughout the S and G2 phases of the cell cycle by Thr-14 and Tyr-15 phosphorylation (11–16). The Wee1 protein kinase phosphorylates Cdc2 on tyrosine 15, whereas the Myt1 protein kinase phosphorylates Cdc2 on both threonine 14 and tyrosine 15 (16–26). In late G2, the Cdc25C phosphatase dephosphorylates Cdc2 on both Thr-14 and Tyr-15, leading to the activation of Cdc2-cyclin B1 complexes (27–31). In addition to cyclin binding and reversible phosphorylation, Cdc2 is also regulated at the level of intracellular compartmentalization. Throughout interphase, Cdc2-cyclin B1 complexes shuttle between the nucleus and the cytoplasm (32–34). The apparent cytoplasmic localization of Cdc2-cyclin B1 complexes (35) is due to a nuclear export signal (NES)1 in cyclin B1 which facilitates rapid export of Cdc2-cyclin B1 complexes from the nucleus. Phosphorylation of the NES in late prophase is proposed to block the nuclear export of cyclin B1 by interfering with the binding of the nuclear export receptor CRM1 leading to the nuclear accumulation of Cdc2-cyclin B1 complexes (33, 36). Thus, entry into mitosis requires not only the activation of Cdc2 by Cdc25C but also the accumulation of active Cdc2-cyclin B1 complexes in the nucleus. Because UCN-01 disrupts G2 checkpoint function by prematurely activating Cdc2, UCN-01 could theoretically interfere with one or more of the Cdc2 regulatory pathways described above.

We undertook a comprehensive study to examine the effects of UCN-01 on proteins that directly regulate Cdc2, including Wee1, Myt1, and Cdc25C. Previously we reported that UCN-01 abrogated G2 arrest following DNA damage through a Cdc2-dependent pathway but did not involve direct inhibition of the Wee1 kinase (4). Indeed, concentrations of UCN-01 as high as 1 μM did not affect the ability of Wee1 to phosphorylate Cdc2-

1 The abbreviations used are: NES, nuclear export signal; GST, glutathione S-transferase; PCR, polymerase chain reaction; ATM, ataxia telangiectasia-mutated; DTT, dithiothreitol; PBS, phosphate-buffered saline; RACE, rapid amplification of cDNA ends; GFP, green fluorescent protein; Gy, gray.

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cyclin B1 complexes in vitro. In the present study, we investigated
effects of UCN-01 on a second Cdc2-inhibitory kinase, Myt1, and on the Cdc25 regulatory pathway.

EXPERIMENTAL PROCEDURES

Chemicals and Reagents—UCN-01 (NSC 638850) was provided by
Jill Johnson (Drug Synthesis and Chemistry Branch, NCI, National
Institutes of Health). Nocodazole (Aldrich) was prepared in dimethyl
sulfoxide.

Mitotic Index—HeLa cells were harvested by trypsinization and then
collected by centrifugation (1000 × g for 5 min). Following washing with
PBS, cells were resuspended in 75 mM KCl for 10 min. Cells were
pelleted and then fixed in acetic acid/methanol (1:3 v/v). Fixed cells
were stained with 0.1 μg/ml 4,6-diamidino-2-phenylindole for 5 min,
allowed to air-dry, and observed by light microscopy. A minimum of 500
cells were counted for each sample.

Cloning of Human Cds1—HeLa total RNA was isolated using the
TRizol reagent RNA isolation kit (Life Technologies, Inc.) followed by
reaction with RNase-free DNase. First strand cDNA was prepared using
1st Strand cDNA Synthesis Kit from CLONTECH. A mixture of
oligo(dT) and random hexamers was used to prime the interrupted
transcription reaction. Degenerate primers were designed to conserved
motifs in the forkhead-associated and kinase domains of Cds1Sp,
Recloned from Drosophila, an ovarian-specific Sec7/Thr kinase from Drosophila
present in GenBank™ (accession number 1848279), and a mouse est
clone (accession number AA762997). Primers 5’-AGTATGAAGGTAA
and 5’-GAGAGGTAA and 5’-GAGAGCTGGGTCTGCCTCTCTTGCTGAA and 5’
were used in combination with the linker primer AP-1 to amplify 5’
and pGEX-2TB, respectively, by digestion with

GST-Chk1 and GST-Cds1 kinase reactions were performed in 50 μl of
kinase reaction buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM
MgCl2, 1 mM DTT, 50 μM cold ATP, 10 μCi of [γ-32P]ATP (3000 Ci
mM⁻¹; NEN Life Science Products), and 5 μg GST-Cdc25C-motif for
10 min at 30 °C. Radiolabeled GST-Cdc25C-motif was excised and radi-
disintegrant quantitated in liquid scintillation counting.

Effect of UCN-01 on Cds25C Serine 216 Phosphorylation and 14-3-3
Interactions—We used either normal HeLa cells or HeLa cells expressing
an inducible human Cdc25C transgene (37) to assess the action of
UCN-01 on Cdc25C-serine 216 phosphorylation and 14-3-3 interaction.
Three million HeLa cells were transfected with Cdc25C(S216A) plasmid,
and were isolated from pGEX-2T using the BAC-TO-BAC Baculovirus Expression System (Life
Institutes of Health). Recombinant baculoviruses were gener-
ated using the BAC-TO-BAC Baculovirus Expression System (Life
Molecular Dynamics).

toradiography kinase reactions were quantified on a PhosphorImager
before being loaded onto 10% SDS-polyacrylamide gels. Following au-
somal washes with PBS and resuspended in 40 ml of His lysis buffer (50
mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% Nonidet P-40, 150 mM sodium
chloride, 1 μM microcin, 1 mM sodium orthovanadate, 2 mM phenyl-
methylsulfonyl fluoride, 0.15 units/ml aprotinin, 20 μM leupeptin,
and 20 μg/ml leupeptin). For normal HeLa cells, total cell lysates were
resolved on an 8% SDS-polyacrylamide gel and immunoblotted with the
Cdc25C monoclonal antibody (174-E10-3) (37). Cdc25C immunoprecipi-
tations were performed from Cdc25C-overexpressing cells with the
same antibody, and the Cdc25C-immune complexes were split and
quantitated by 7% SDS-polyacrylamide gels for immu-
nonblotting with Cdc25C (174-E10-3) or 14-3-3 (K-19, Santa Cruz
Biotechnology) antibodies, respectively. Cdc2 and cyclin B1 immuno-
 blotting of total cell lysates was performed using the Cdc2 antibody
(SC-54) and cyclin B1 antibody (SC-245) (Santa Cruz Biotechnology).
Bound primary antibodies were detected using the ECL (Amersham
Pharmacia Biotech) detection system.

Effect of UCN-01 on Cds1 Phosphorylation in Response to DNA
Damage—HeLa cells were treated with different concentrations of
UCN-01 for 1 h before subjecting them to 0, 5, 10, or 20 Gy of gamma
rays. At 2 h post-irradiation, cells were harvested and lysed in mamma-

Generation and Purification of Human Cds1 Antibody—BL21 cells
were transformed with pET15b-huCds1. Cultures (1 liter) were grown
at 30 °C to an A600 of 0.5, and isopropyl-1-thio-β-D-galactopyranoside
was added to a final concentration of 0.5 mM. After growing for an
additional 4 h at 30 °C, cells were pelleted by centrifugation. Cell pellets
were washed with PBS and resuspended in 40 ml of His buffer (50
mM Tris-HCl, pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, 1
mM mg/μl lysosome) supplemented with protease inhibitors (2 mM phenyl-
methylsulfonyl fluoride, 0.15 units/ml aprotinin, 20 μM leupeptin,
and 20 μg/ml leupeptin). After rocking at 4 °C for 15 min, cells were lyzed by sonica-
tion (50% duty for 15 bursts). The lysate was centrifuged (10,000 × g for 15 min at 4 °C) and mixed with 2 ml of packed Ni-NTA-agarose (Qiagen) for 1 h at 4 °C. The beads were washed 3 times
with 10 ml of wash buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5
mM EDTA, 0.5% Nonidet P-40) and transferred to a Bio-Rad dispos-
able column. Protein was eluted with elution buffer (50 mM Tris-HCl,
ph 8.0, 150 mM NaCl, 100 mM imidazole, 1 mM DTT, 0.1% Nonidet
P-40), and purified His-huCds1 protein was injected into New Zealand
White rabbits. To purify antibodies specific for huCds1, GST-huCds1
P-40), and purified His-huCds1 protein was coupled to Affi-Gel 10 (Bio-Rad) according to the manufacturer's
instructions. After flowing huCds1 antiserum over the column and washing,
antibodies were eluted with 0.1 μM glycine, pH 2.8, and imme-
diately neutralized with 1 M Tris, pH 8.0.

Effects of UCN-01 on the Localization of Cdc25C—HeLa cells were
grown on 12-mm glass coverslips and transfected with the plasmids
pEGFP-Cdc25C or pEGFP-Cdc25C(S216A) using 20 μg/100-mm dish
and the calcium phosphate transfection system according to the manu-
facturer's instructions (Life Technologies, Inc.). At 24 h post-transfection,
cells were fixed with 2% paraformaldehyde in PBS. After washing 3
times in PBS, cells were mounted on slides using the Prolong Antifade
kit (Molecular Probes, Eugene OR). Cells were viewed using a confocal
laser microscope (MRC 1024, Bio-Rad). In some cases, 300 nM UCN-01
was added during the last 2 h of the transfection.

Mutational analysis of UCN-01 in vitro—Because UCN-01 is a protein kinase inhibitor, we first investigated
whether UCN-01 was promoting mitotic entry in DNA-dam-

plasmids—The coding sequence of full-length huCds1 was amplified by
PCR using the following two primers: 5’-CCCGAATTCATTATGTTCTGC
AGTCACAACACAGCAGCACACAC (EcoRI/XhoI primer). The PCR
product was ligated to the TA cloning vector, and the resulting plasmid
pTA-huCds1 was digested with Ndel and EcoRI. The fragment encoding
huCds1 was inserted into the corresponding sites of pEGEX2NT to create
pEGEX2NT-huCds1. pTA-huCds1 was digested with Ndel and XhoI and
cloned into the corresponding sites of PET15b to generate PET15b-
huCds1. pEGEX2NT-huCds1 was digested with XbaI and EcoRI, and the
fragment encoding huCds1 was cloned into the Stul site of pFASTBAC
to create pFASTBAC-huCds1. Recombinant baculoviruses were gener-
ated using the BAC-TO-BAC Baculovirus Expression System (Life
Technologies, Inc.) and protocols suggested by the manufacturer. Wild-
type human Cdc25C and Cdc25C(S216A) were isolated from pEGEX-2T
and pEGEX-2TB, respectively, by digestion with BamHI and cloned into
the BamHI site of pEGFSP-1 to create pEGFP-Cdc25C and
pEGFP-Cdc25C(S216A).

Protein Kinase Assays—GST-Chk1, GST-Cds1, His-Myt1, GST-
Cdc25C-motif, and GST-Cyclin B1/Cdc2/RUR) were isolated from insect
cells or bacteria as described previously (4, 16, 23, 37). His-Myt1 kinase
reactions were carried out with GST-cyclin B1/Cdc2/RUR) at 30 μl of
reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 1 mM DTT, 50
μM cold ATP, 10 μCi of [γ-32P]ATP (3000 Ci
mM⁻¹; NEN Life Science Products) for 10 min at 30 °C. Reactions were terminated by adding an
equal volume of 2× SDS sample loading buffer and boiling for 5 min before being loaded onto 10% SDS-polyacrylamide gels. Following au-
toradiography kinase reactions were quantitated on a PhosphorImager
(Molecular Dynamics).
Chk1 and Cdc25C as Targets of UCN-01 Action

The Cdc25C Regulatory Pathway Is Inhibited by UCN-01 in Vivo—We next examined the effect of UCN-01 treatment on the intracellular localization of Cdc25C because 14-3-3 binding is not the sole determinant regulating Cdc25C intracellular compartmentalization. A nuclear export sequence in Cdc25C likely contributes to the prominent cytoplasmic staining of both Cdc25C and of the Cdc25C(S216A) mutant protein.² Because UCN-01 treatment causes loss of 14-3-3 binding to Cdc25C, the fluorescence pattern of wild-type Cdc25C in the presence of UCN-01 was predicted to be similar to that of Cdc25C(S216A). This was indeed the case as GFP-Cdc25C was detected in both the nucleus and the cytoplasm of cells treated with UCN-01 (bottom left panel). The prominent cytoplasmic staining of Cdc25C in the presence of UCN-01 indicates that UCN-01 does not perturb Cdc25C NES function. In addition, UCN-01 treatment did not detectably alter the localization of Cdc25C(S216A) (bottom right panel). Although UCN-01-treated cells showed only modest nuclear accumulation of Cdc25C, this may be sufficient to initiate the autocatalytic loop that has been described for Cdc2 activation and mitotic entry (41, 47, 48) (see “Discussion”).

To determine the concentration of UCN-01 where loss of Cdc25C-serine 216 phosphorylation could first be observed, HeLa cells were incubated with varying concentrations of UCN-01, and the electrophoretic mobility of Cdc25C was monitored by immunoblotting (Fig. 2C). Serine 216 dephosphorylation was monitored by observing the accumulation of species a after 2 h of incubation with UCN-01. We found that Cdc25C was dephosphorylated on serine 216, as indicated by an increase in band a, with as little as 10 nM UCN-01. Although the majority of Cdc25C was not dephosphorylated until UCN-01

Fig. 1. Effect of UCN-01 on Myt1 kinase activity in vitro and on γ-ray-induced G₂ arrest in HeLa cells. A, His-Myt1 was purified from insect cells and incubated in a kinase reaction that contained varying concentrations of UCN-01 and an inactive version of Cdc2 (K33R) complexed with GST-cyclin B1 as a substrate. Following autoradiography, kinase reactions were quantified by determining the amount of radiolabel incorporated into the Cdc2(K/R) band using a PhosphorImager (Molecular Dynamics). B, HeLa cells were incubated for 12 h following 6 Gy of gamma rays in the presence of 0.15 μg/ml nocodazole to trap any cells that entered mitosis. UCN-01 was then added to the culture, and cells were incubated for an additional 8 h to allow for mitotic entry before harvesting and performing mitotic index measurements. The dotted line at 100% represents the normalized percent of cells that accumulated in mitosis upon incubation with nocodazole alone for 20 h (~80% of total culture). The dotted line at 0% represents the normalized percent of cells that accumulated in mitosis following irradiation and incubation with nocodazole (~11% of total culture).

AGED cells through direct inhibition of the Cdc2-inhibitory kinase, Myt1 (25, 26, 38, 39). We assayed purified Myt1 in vitro in the presence of UCN-01 (Fig. 1A) and found that Myt1 was only weakly inhibited by UCN-01. Furthermore, the concentration of UCN-01 required to inhibit Myt1 activity by 50% (IC₅₀) was significantly higher than that required to abrogate G₂ arrest following gamma irradiation of HeLa cells (IC₅₀ ~15–20 nM) (Fig. 1B). Taken together, these and previously published (4) results suggested that Wee1 and Myt1 were not the primary targets through which UCN-01 abrogated DNA-damaged-induced G₂ arrest.

The Cdc25C Regulatory Pathway Is Inhibited by UCN-01 in Vivo—We next examined whether UCN-01 targeted the Cdc25C regulatory pathway. Cdc25C is negatively regulated by phosphorylation of serine 216 throughout interphase and upon G₂ checkpoint activation (37). During interphase, Cdc25C migrates in SDS gels as two distinct electrophoretic forms, a slower migrating serine 216-phosphorylated form (species b, see Fig. 2A) that binds 14-3-3 proteins (37) and a faster migrating hypophosphorylated form (species a). In mitosis, Cdc25C becomes hyperphosphorylated which reduces its electrophoretic mobility further (species c) (37, 40, 41). Throughout interphase and in the presence of unreplicated or damaged DNA, Cdc25C is phosphorylated on serine 216 and bound to 14-3-3 proteins (37). To determine whether UCN-01 treatment disrupted the Cdc25C regulatory pathway, HeLa cells induced to express Cdc25C (37) were subjected to gamma irradiation and then incubated with or without UCN-01 in the presence of nocodazole to trap any cells entering into mitosis (Fig. 2A). In the absence of UCN-01, Cdc25C was found to be predominantly in the serine 216-phosphorylated form (species b) and bound to 14-3-3 proteins. Mitotic index measurements demonstrated that cells maintained their G₂ cell arrest under these conditions indicative of an intact checkpoint response. In contrast, UCN-01 addition caused the loss of Cdc25C-serine 216 phosphorylation (within 1 h) and the Cdc25C-14-3-3 complex disassembled. These events preceded Cdc2 dephosphorylation and entry of cells into mitosis at which time Cdc25C became hyperphosphorylated (species c).

UCN-01 Modulates the Localization of Cdc25C in Vivo—We next examined the effect of UCN-01 treatment on the intracellular localization of Cdc25C because 14-3-3 binding has been shown to contribute to the nuclear exclusion of Cdc25 (42–46). Cdc25C and a mutant of Cdc25C that cannot bind to 14-3-3 proteins (Cdc25C(S216A)) (37) were tagged with the green fluorescent protein (GFP) and transiently expressed in HeLa cells (Fig. 2B). In the absence of UCN-01, GFP-tagged wild-type Cdc25C localized exclusively to the cytoplasm (top left panel), whereas both nuclear and cytoplasmic fluorescence was detected in cells expressing GFP-tagged Cdc25C(S216A) (top right panel). These findings are consistent with those reported by Dalal et al. (46). A large fraction of Cdc25C(S216A), the 14-3-3 binding mutant of Cdc25C, was detected in the cytoplasm indicating that 14-3-3 binding is not the sole determinant regulating Cdc25C intracellular compartmentalization. A nuclear export sequence in Cdc25C likely contributes to the prominent cytoplasmic staining of both Cdc25C and of the Cdc25C(S216A) mutant protein.² Because UCN-01 treatment causes loss of 14-3-3 binding to Cdc25C, the fluorescence pattern of wild-type Cdc25C in the presence of UCN-01 was predicted to be similar to that of Cdc25C(S216A). This was indeed the case as GFP-Cdc25C was detected in both the nucleus and the cytoplasm of cells treated with UCN-01 (bottom left panel). The prominent cytoplasmic staining of Cdc25C in the presence of UCN-01 indicates that UCN-01 does not perturb Cdc25C NES function. In addition, UCN-01 treatment did not detectably alter the localization of Cdc25C(S216A) (bottom right panel). Although UCN-01-treated cells showed only modest nuclear accumulation of Cdc25C, this may be sufficient to initiate the autocatalytic loop that has been described for Cdc2 activation and mitotic entry (41, 47, 48) (see “Discussion”).

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² P. R. Graves and H. Piwnica-Worms, unpublished observations.
reached 300 nM, it is conceivable that a small amount of dephosphorylated Cdc25C may be able to initiate the autocatalytic loop and contribute to bypass of the arrest.

UCN-01 Potently Inhibits the Kinase Activity of Chk1 but Not Cds1 in Vitro—We and others (37, 49–53) have previously demonstrated that the Chk1 and Cds1 protein kinases phosphorylate human Cdc25C on serine 216 in vitro. We performed kinase assays to assess the inhibitory activity of UCN-01 on the human Chk1 and Cds1 protein kinases (Fig. 3A). We used a region of Cdc25C (amino acids 200–256) encompassing the site where Chk1 and Cds1 phosphorylate Cdc25C (serine 216) as a substrate. UCN-01 was a potent inhibitor of Chk1 autophosphorylation (data not shown) and Cdc25C-serine 216 phosphorylation with IC50 values of 3.25 nM (Fig. 3A). Collectively, these data are consistent with Chk1 being a target of UCN-01 action in vivo. In contrast, Cds1 retained 84% of its activity in the presence of 1 μM UCN-01 indicating that Cds1 is not likely to be a direct target of UCN-01 action. UCN-01 interfered with the DNA damage-induced phosphorylation of Cds1 in vivo. This phosphorylation is dependent upon ATM function and reflects activation of Cds1 in vivo following...
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DNA damage. HeLa cells incubated with UCN-01 were irradiated, and the phosphorylation status of Cds1 was determined by monitoring the mobility of Cds1 on SDS gels. UCN-01 treatment had no effect on the ability of Cds1 to become phosphorylated in response to DNA damage (Fig. 3B, top panel) even with concentrations of UCN-01 that resulted in significant loss of Cdc25C-serine 216 phosphorylation (bottom panel). Although the results shown in Fig. 3B were obtained using 20 Gy of γ-irradiation, similar results were obtained with 5 and 10 Gy (data not shown). Because the mobility shift of Cds1 observed in the presence of DNA damage is dependent on a functional ATM protein, we infer that UCN-01 does not significantly inhibit the activity of ATM in vivo.

DISCUSSION

The G2 DNA damage checkpoint operates at least in part by preventing the Cdc25 phosphatase from activating the Cdc2 kinase. This is accomplished by maintaining Cdc25 in a phosphorylated form that binds 14-3-3 proteins (37, 43, 54, 55). The binding of 14-3-3 proteins prevents Cdc25 from accumulating in the nucleus (42–46), and this presumably prevents Cdc2 from being activated in the nucleus as it shuttles between the cytoplasm and the nucleus during the G2 checkpoint response. Our results establish an important link between UCN-01, an agent undergoing clinical trials for cancer treatment, and the Cdc25C regulatory pathway (Fig. 4). We report that treatment of cells with UCN-01 causes Cdc25C to become dephosphorylated on serine 216. Consistent with this phosphorylation site being important for checkpoint fidelity, mutation of serine 216 to alanine attenuates checkpoint control in human cells (37). The Chk1 protein kinase phosphorylates Cdc25C on serine 216 (49), and this kinase is particularly sensitive to inhibition by UCN-01. These results provide a formal link between the G2 checkpoint abrogating activity of UCN-01, the Cdc25C regulatory pathway, and the Chk1 protein kinase (Fig. 4).

Although a pivotal role for Chk1 in controlling the DNA damage G2 checkpoint has been demonstrated in fission yeast (56, 57), a similar role for the human Chk1 kinase has not been demonstrated. Interestingly, Chk1 appears to play a supportive role to the Cds1 kinase in regulating the DNA replication checkpoint in fission yeast (54, 58, 59). Both Chk1 and Cds1 phosphorylate fission yeast Cdc25 at sites that enable 14-3-3 protein binding, and mutation of these sites in Cdc25 disrupts both the DNA replication and damage checkpoints in fission yeast (43, 54). Consistent with the fission yeast findings, the human Chk1 and Cds1 protein kinases phosphorylate Cdc25C on serine 216, the 14-3-3-binding site (49–53). Although the human Chk1 and Cds1 protein kinases phosphorylate Cdc25C on serine 216 in vitro, there is no functional data to demonstrate that these kinases function to phosphorylate human Cdc25C in vivo. Our results indicate that Chk1 may indeed function as a Cdc25C kinase in vivo. In contrast, the finding that the Cdc25C regulatory pathway is disrupted in UCN-01-treated cells, yet the Cds1 pathway is intact, may indicate that Cds1 does not serve as a major Cdc25C kinase in DNA-damaged cells (Fig. 4).

Entry into mitosis requires both the activation of Cdc2 by Cdc25C and the accumulation of active Cdc2 within the nucleus of the cell. Although it is unclear how the interactions between Cdc2 and Cdc25 are initiated, one requirement may be the dephosphorylation of Cdc25C on serine 216 and subsequent loss of 14-3-3 binding. Loss of 14-3-3 binding allows Cdc25C to move into the nucleus more efficiently and in turn enhances the accessibility of Cdc25C to nuclear pools of Cdc2-cyclin B1. Once a small amount of Cdc2 became activated by Cdc25C, an auto-amplification loop would be initiated whereby activated Cdc2 promotes the activation of Cdc25C which in turn triggers further activation of Cdc2. This pathway coupled to the one that down-regulates the Cdc2 inhibitory kinases provides a mechanism for the rapid amplification of Cdc2 activity observed as cells enter into mitosis (41, 47, 48). Thus, UCN-01 may cause efficient activation of Cdc2 by causing only partial dephosphorylation of Cdc25C on serine 216. Consistent with this hypothesis, at the IC50 of UCN-01 required for abrogating G2 arrest (15–20 nM), a modest but not complete dephosphorylation of Cdc25C on serine 216 was observed (Fig. 2C).

UCN-01 must affect pathways in addition to the Cdc25C-serine 216 regulatory pathway because the checkpoint abrogation observed after UCN-01 treatment is much more severe than that observed after expression of a 14-3-3-binding mutant of Cdc25C (37). The fact that cells treated with checkpoint abrogators like UCN-01 cause premature entry into mitosis indicates that active Cdc2 is accumulating in the nucleus under these conditions. Disruption of the Cdc25C regulatory pathway can explain how Cdc2 becomes activated but does not address how active Cdc2 then accumulates in the nucleus. Throughout interphase and during the cellular response to DNA damage, Cdc2-cyclin B1 complexes shuttle between the nucleus and the cytoplasm (32–34). The apparent static cytoplasmic localization of Cdc2-cyclin B1 complexes is due to a nuclear export sequence in cyclin B1 that facilitates nuclear export of these complexes. Cyclin B1 NES function is regulated by phosphorylation, and therefore kinases in the pathway regulating cyclin B1 NES function could also be potential targets of UCN-01 (33, 36). Finally, although UCN-01 does not directly inhibit the kinase activity of Wee1, it may interface with upstream regulators of Wee1 and thereby inhibit the Wee1 regulatory pathway (4, 60).

The p53 tumor suppressor is essential for DNA-damaged cells to arrest at the G1 checkpoint (61, 62). Mutations in p53, a common occurrence in human cancer, abolish this response and in turn leave cancer cells vulnerable to abrogation of the last remaining barrier protecting them from the cytotoxicity of DNA-damaging agents, the G2 checkpoint. Drugs designed to abrogate G2 checkpoint function by inhibiting the function of the Chk1 kinase could improve the killing of p53-defective tumors and as such exhibit broad utilization in cancer chemo-
therapeutic and radiotherapy regimens (63). Indeed, observations that cancer cells deficient in p53 tumor suppressor function are more vulnerable to G2 checkpoint abrogation (1, 64, 65) suggest this strategy might prove selective to cancer cells.

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