Abstract

The investigational anticancer agent 7-hydroxystaurosporine (UCN-01) abrogates the G2 checkpoint in tumor cells and sensitizes them to the lethal effects of genotoxic anticancer agents. On the basis of the role of the Cdc25C phosphatase in maintenance of this damage-inducible checkpoint, we hypothesized that UCN-01 inhibits a component of the signal transduction pathway that modulates Cdc25C phosphorylation. Of the three kinases known to phosphorylate Cdc25C on Ser112, both checkpoint kinase 1 (hChk1) and Cdc25C-associated protein kinase 1 (cTAK1) were potently inhibited by UCN-01 with IC50 of 11 and 27 nM, respectively. Treatment of K562 erythroleukemia leukemia cells with similar drug concentrations resulted in decreased levels of Ser216 phosphorylation of Cdc25C and complete disruption of the γ-radiation-induced G2 checkpoint. In contrast to hChk1, the hChk2 kinase was 100-fold more resistant to inhibition by UCN-01 (IC50 1040 nM). These results suggest that disruption of the DNA damage-induced G2 checkpoint by UCN-01 is mediated through the inhibition of the Cdc25C kinases, hChk1 and cTAK1, and that hChk2 activity is not sufficient to enforce the G2 checkpoint in cells treated with a pharmacological inhibitor of hChk1.

Introduction

DNA damage-inducible cell cycle checkpoints are complex signal transduction networks that integrate the cellular responses to genotoxic insults by arresting cell cycle progression during the repair of DNA damage or the induction of apoptosis. The integrity of these checkpoint pathways is critical for maintenance of genomic stability and cellular recovery from genotoxic damage. In genetic models, loss of the G2-M checkpoint response might be useful in cancer therapy as radiomodulating and chemosensitizing agents (1). In fact, UCN-01 is one of several compounds that abrogates the G2-M checkpoint at concentrations associated with sensitization of cells to DNA-damaging agents (2,3). Because UCN-01 inhibits multiple protein kinases (4), the identification of the molecular target(s) responsible for UCN-01-mediated checkpoint abrogation could facilitate the identification of more selective second-generation checkpoint inhibitors.

The induction of a G2 arrest after DNA damage depends, in part, on inhibition of cyclin B1/Cdk1 activity through phosphorylation of the Cdk1 subunit at Thr14 and Tyr15 (5). The dynamic changes in the phosphorylation of Cdk1 during both the normal cell cycle and in response to DNA damage are controlled by an interplay between the enzymatic activities of the Wee1-like kinases and the Cdc25C phosphatase. In recent years, the signaling pathways controlling Cdc25C activation and subcellular localization have been partially elucidated. Current models suggest that the serine-threonine kinases hChk1 and hChk2 are activated by DNA damage signaling pathway(s) dependent on the ATM family of protein kinases (6–8). Activated hChk1 and/or hChk2 then phosphorylate Cdc25C on Ser216, which creates a consensus binding site for 14-3-3 proteins. In undamaged interphase cells, Ser216 is also phosphorylated by a third, constitutively active kinase, the Cdc25C-associated protein kinase (cTAK1). Association with 14-3-3 proteins leads to sequestration of phosphorylated Cdc25C in the cytoplasm and prevents premature dephosphorylation of Cdk1 and activation of the cyclin B1/Cdk1 complex.

Staurosporine and UCN-01 are capable of inhibiting the phosphotransferase activities of several serine-threonine protein kinases (4). Because the kinase activity of Weel is resistant to UCN-01 (9), we hypothesized that the mechanism of UCN-01-mediated checkpoint abrogation might involve inhibition of one or more of the kinases that regulate Cdc25C. In this study, we show that UCN-01 inhibits hChk1 and cTAK1 at drug concentrations associated with abrogation of the G2 checkpoint. In contrast, hChk2 was relatively resistant to checkpoint inhibitory concentrations of UCN-01. These data identify hChk1 as a relevant molecular target for UCN-01 and suggest that specific hChk1 inhibitors will efficiently disrupt the G2 checkpoint.

Materials and Methods

Cell Culture, Antibodies, and Plasmid Constructs. The K562 erythroleukemia leukemia cell line was maintained in RPMI 1640 (Life Technologies, Inc.) containing 10% fetal bovine serum. UCN-01 (generously provided by Dr. Edward Saussville, National Cancer Institute) was dissolved in DMSO and stored at –20°C. The viral HA-specific mouse monoclonal antibody, HA.11, was purchased from Babco, and a Cdc25C-specific polyclonal rabbit antiserum (C-20) was purchased from Santa Cruz Biotechnology. hChk1 and hChk2 were amplified by PCR from a human testes cDNA library (Clontech), and the respective full-length cDNA was cloned into the pEF-BOS plasmid with COOH-terminal HA epitope tags (HA3). cTAK1 was amplified from the same cDNA library and cloned into the pcDNA3.1+ plasmid with an NH2-terminal tandem HA epitope tag. The coding sequence for cTAK1 differed from that published previously and contained a single amino acid substitution (G443S; Ref. 10). This difference was present in all five cDNA clones sequenced and in nine human sequences from the dbEST database (GenBank), which suggests that this variant represents a polymorphism of the cTAK1 sequence. The kinase dead point-mutants, Chk1 (D130A), Chk2 (D347A), and cTAK1 (D196N), were generated by site-directed mutagenesis using the GeneEditor kit from Promega Corp. The GST–Cdc25C595–256 fusion protein was generated as described previously (11).

Immunocomplex Kinase Assays. The hChk1 and hChk2 kinase assays were performed as described previously for hChk1 (11). Kinase assays for cTAK1 were performed essentially as described for hChk1 except for minor changes in the immunoprecipitation conditions. K562 cells transiently expressing epitope-tagged cTAK1 were lysed in buffer (20 mM HEPES, 0.15 mM NaCl,
1.5 mM MgCl₂, and 1 mM EDTA, pH 7.4) containing 1 mM DTT, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 20 mM microcystin, 10 mM β-glycerophosphate, and 0.5% Triton X-100. After immunoprecipitation with HA.11 and protein A-Sepharose beads, immunoprecipitates were washed twice in lysis buffer, twice in high-salt buffer [0.1 M Tris-HCl (pH 7.4), 0.6 M NaCl], and twice in kinase buffer [50 mM Tris (pH 7.4), 10 mM MgCl₂]. Kinase assays were then performed as outlined previously for hChk1 (11). All kinase reactions were performed under linear reaction conditions. 

**Flow Cytometry.** Ethanol-fixed cells were stained with propidium iodide and analyzed by flow cytometry as described previously (12).

**Cdc25C Mobility.** K562 cells were treated with graded concentrations of UCN-01 and then lysed in TNE buffer [50 mM Tris (pH 8.0), 150 mM sodium chloride, 5 mM EDTA, 1% NP40, and 0.1% SDS] containing 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 20 mM microcystin, 200 μM dephostatin, 10 μM cypermetrin, 200 μM okadaic acid, and 25 μM tautomycin. The detergent-soluble proteins were then processed for immunoblotting with Cdc25C-specific antisera.

**hChk1/hChk2 Mobility Shift.** K562 cells were lysed in TNE buffer containing 1 mM DTT, 10 μg/ml aprotinin, 5 μg/ml pepstatin, 5 μg/ml leupeptin, 20 mM microcystin, and 10 mM β-glycerophosphate. The detergent-soluble proteins were then processed for immunoblotting with the HA.11 monoclonal antibody.

**Statistics.** All statistical analyses were performed with the Sigma Plot 5.0 (SPSS) software package. The kinase inhibition data were fit with the Hill four-parameter model by the least-squares method. The concentrations of UCN-01 resulting in half-maximal inhibition (IC₅₀) for the respective kinases were calculated by solving the model for a relative activity of 0.5.

**Results and Discussion.**

As a preliminary step toward identifying relevant molecular target(s) inhibited by UCN-01, we developed a UCN-01 concentration-inhibition relationship for disruption of the G₂ checkpoint in exponentially growing K562 erythroleukemia leukemia cells. K562 cells were treated with 8 Gy γ-radiation and graded concentrations of UCN-01. After 21 h, ~80% of cells were arrested in G₂, consistent with the lack of a DNA damage-inducible G₁ checkpoint in this p53-null cell line. In contrast, treatment of cells with 100 nM UCN-01 completely abrogated the radiation-induced G₂ arrest (Fig. 1A). When parallel samples were treated with the microtubule inhibitor nocodazole, nearly all irradiated cells were arrested with a tetraploid DNA content with or without UCN-01 treatment (Fig. 1A and data not shown). These results demonstrate that the drug-treated cells traversed the G₂-M checkpoint and were not simply arrested in G₁. On the basis of these data, we predicted that one or more kinases in the G₂ checkpoint pathway would be inhibited by UCN-01 at concentrations of ≤100 nM.

The phosphorylation of Cdc25C on Ser²¹⁶ is an important upstream event during the induction of a G₂ arrest in cells exposed to DNA-damaging agents. Three protein kinases (hChk1, hChk2, and cTAK1) are thought to be capable of phosphorylating the Ser²¹⁶ site in mammalian cells. To determine which, if any, of these protein kinases might be relevant targets inhibited by UCN-01, we examined the sensitivity of these kinases to UCN-01 in immune-complex kinase assays. Epitope-tagged, wild-type, or kinase-dead (D130A) hChk1 was immunoprecipitated from transiently transfected K562 cells, and immune complex kinase assays were performed with a GST-Cdc25C fusion protein as a substrate. Wild-type hChk1 phosphorylated GST-Cdc25C²⁰⁰–²⁵⁶ to a significantly higher level than the background activity present in the kinase-dead control, and coincubation with 300 nM UCN-01 reduced wild-type hChk1 kinase activity to background (Fig. 2A). When the concentration of UCN-01 was varied, an IC₅₀ of 11 nM was observed. In previous work evaluating the effects of UCN-01 on PKC, there was little difference in the concentrations of UCN-01 required to inhibit PKC kinase activity in vitro and a PKC-dependent signaling pathway in intact cells (13). This suggests that the intracellular concentration of UCN-01 approaches that present in the surrounding medium. Thus, the close correlation between the effects of UCN-01 on the integrity of the G₂ checkpoint in intact cells and on hChk1 kinase activity in vitro suggests that inhibition of hChk1 may contribute to UCN-01-mediated checkpoint disruption.

Recent studies in mammalian cells have demonstrated that γ-radiation-induced hChk2 activation requires ATM kinase activity and that the kinase activity of hChk2 was 100-fold more resistant to the inhibitory effects of UCN-01 than that of hChk1 (Fig. 2B). Other studies combining radiation and UCN-01 treatment showed that the kinase activity of hChk2 was 100-fold more resistant to the inhibitory effects of UCN-01 than that of hChk1. Our data suggest that concomitant inhibition of both hChk1 and hChk2 kinase activities is not required for G₂ checkpoint abrogation.

The phosphorylation of Cdc25C on Ser²¹⁶ and the exclusion of
Cdc25C from the nucleus are maintained throughout interphase. In unstressed cells, this phosphorylation is presumably maintained by the cTAK1 kinase (10). We evaluated the sensitivity of cTAK1 to UCN-01 to complete our survey of known protein kinases that can phosphorylate Cdc25C on Ser216. Interestingly, cTAK1 was nearly as sensitive as hChk1 to the inhibitory effects of UCN-01 in immune-complex kinase assays with an IC50 of 27 nM (Fig. 2C). Although cTAK1 has no known function in the DNA damage checkpoint responses, we cannot exclude the possibility that inhibition of cTAK1 kinase activity by UCN-01 could contribute to the checkpoint inhibitory effects of this drug.

The results presented, to this point, support the hypothesis that the checkpoint inhibitory effects of UCN-01 are related to the inhibition of hChk1 but not hChk2 kinase activities. Both hChk1 and hChk2 are inducibly phosphorylated in response to genotoxic agents, and these modifications are presumed to be important in checkpoint activation (6, 16). Therefore, a UCN-01-induced block in an upstream event modulating hChk1 or hChk2 activation also might lead to abrogation of the G2 checkpoint. To evaluate the effects of UCN-01 on the integrity of these upstream signaling pathways, we assessed whether UCN-01 affected the DNA damage-induced phosphorylation of transiently expressed hChk1 and hChk2 in K562 cells treated with 10 mM HU or 20 Gy γ-radiation. The mobility of hChk1 on SDS-PAGE was appreciably retarded 1 h after irradiation or continuous exposure to HU (data not shown), and pretreatment with 1 μM UCN-01 had no effect on this hChk1 mobility shift (Fig. 3A). Likewise, the radiation-induced mobility shift of hChk2 also was unchanged by UCN-01 pretreatment (Fig. 3B). Consistent with this observation, we have shown previously that the ATM kinase, which functions directly upstream of hChk2, also is resistant to inhibition by UCN-01 (11). Thus, in contrast to the protein kinase activity of hChk1 itself, the pathways leading to hChk1 and hChk2 activation in damaged cells are not sensitive to UCN-01.

The maintenance of Cdc25C phosphorylation on Ser216 after DNA damage is thought to prevent premature progression from G2 into mitosis. Throughout interphase, the vast majority of Cdc25C is phosphorylated on Ser216 (17). This modification can be detected by the reduced mobility of Cdc25C on SDS-PAGE (18). Because UCN-01 inhibits two protein kinases that modify the regulatory Ser216 site, we hypothesized that UCN-01 might affect the phosphorylation status of Cdc25C in irradiated cells. To test this, K562 cells were exposed to 12 Gy γ-radiation. After 16 h, 95% of cells were arrested in G2. Treatment of this G2-arrested cell population with 100 nM UCN-01 resulted in a significant increase in the nonphosphorylated, relative to the phosphorylated, form of Cdc25C within 15 min of drug addition. Moreover, graded concentrations of UCN-01 resulted in considerable accumulation of the nonphosphorylated Cdc25C species (Fig. 3C) at
concentrations associated with significant inhibition of hChk1 and cTAK1 kinase activities (Fig. 2, A and C). In combination with our cell cycle data (Fig. 1B), these results are consistent with loss of G2 checkpoint integrity at UCN-01 concentrations associated with marked inhibition of intracellular hChk1 and cTAK1 kinase activities.

The ATM and ATR proteins appear to control downstream signaling events in the G2 checkpoint pathway (19–21). We have shown previously that the checkpoint inhibitory effects of the radiosensitizing agent caffeine were related to the inhibition of ATM and ATR kinase activities (11). Both epistasis experiments in yeast and genetic studies in mammalian cells suggest that these protein kinases participate in the activation of hChk1 and hChk2, which can then phosphorylate downstream targets such as Cdc25C. The present findings suggest that inhibition of hChk1 and cTAK1 kinase activities is sufficient to abrogate the G2 checkpoint. Strikingly, the relative resistance of the hChk2 kinase to inhibition by UCN-01 suggests that activation of the hChk2 kinase, in the absence of hChk1 and cTAK1 kinase activities, is insufficient to maintain the γ-radiation-induced G2 arrest. This implies either that phosphorylation of Cdc25C by hChk2 alone is insufficient to arrest cell cycle progression or that hChk2 is not a physiologically relevant protein kinase for Cdc25C in DNA-damaged cells. Although we cannot exclude the potential role for cTAK1 in the induction and/or maintenance of the checkpoint, our results reinforce the notion that a specific inhibitor of hChk1 would be an effective inhibitor of the G2 checkpoint.

Recent evidence suggests that the therapeutic efficacies of certain DNA-damaging anticancer drugs are causally related to the loss of normal DNA damage checkpoint controls during the process of carcinogenesis (22, 23). These studies suggest that agents that interfere with checkpoint-related proteins may show selectivity for tumor cells bearing intrinsic defects in specific checkpoint pathways. One of the most common mutations that affects checkpoint integrity is inactivation of the p53 tumor suppressor protein. Because p53 functions in several checkpoint pathways (19), the loss of p53 might leave tumor cells more vulnerable to pharmacological inhibition of components of the remaining checkpoints in these cells. The expected outcome of checkpoint inhibitor treatment in this setting would be a selective increase in the sensitivities of cancer cells to conventional genotoxic cancer therapies. In fact, both caffeine and UCN-01 selectively abrogate the G2 checkpoint in tumor cells that have lost p53-dependent checkpoint controls (2, 24–26). The recent demonstration that homozygous deletion of 14-3-3σ in HCT116 colon cancer cells compromises the G2 checkpoint provides novel insight into a potential mechanism for the selective pharmacological inhibition of the G2 checkpoint (Fig. 4). In normal cells, DNA damage leads to a p53-dependent accumulation of 14-3-3σ, which binds to and sequesters cyclin B1/Cdk1 complexes in the cytoplasm (27). With cyclin B1/Cdk1 excluded from the nucleus, pharmacological disruption of the hChk1-dependent checkpoint pathway controlling Cdc25C localization will not lead to premature entry into mitosis. However, in tumor cells deficient in p53 function, the integrity of the G2 arrest is solely dependent on the hChk1-dependent pathway, and disruption of this pathway could then lead to checkpoint abrogation.

Acknowledgments

We thank Drs. Scott Kaufmann and Junjie Chen for helpful advice and discussions and James Tarara and the other members of the Mayo Cancer Center Flow Cytometry Laboratory for expert technical assistance.

Note Added in Proof

After the resubmission of our revised manuscript, Graves et al. (P. R. Graves, J. Biol. Chem., 275: 5600–5605, 2000) reported that UCN-01 potently inhibits the kinase activity of hChk1 (IC50 25 nM) but not hChk2.

References


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