Abrogation of Chk1-mediated S/G2 checkpoint by UCN-01 enhances ara-C-induced cytotoxicity in human colon cancer cells[1]

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ABSTRACT

AIM: To investigate whether 7-hydroxystaurosporine (UCN-01) affects cell cycle progression in arabinosylcytosine (ara-C) treated human colon carcinoma HT-29 cells. **METHODS**: Cytotoxicity, DNA synthesis, cell cycle distribution, protein level, and kinase activity were determined by clonogenic assay, flow cytometry, DNA synthesis assay, immunoblotting, and kinase assays, respectively. **RESULTS**: UCN-01 abrogated an S/G2-phase checkpoint in HT-29 cells treated with ara-C. When UCN-01 was added after treatment with ara-C, the rate of recovery of DNA synthesis was enhanced and colony-forming ability diminished. Thus, premature recovery of DNA synthesis was associated with increased cytotoxicity. Measurements of cyclin A and B protein levels, Cdk2 and Cdc2 kinase activities, Cdc25C phosphorylation, and Chk1 kinase activity were consistent with UCN-01-induced abrogation of the S/G2-phase checkpoint in ara-C treated cells. **CONCLUSION**: The abrogation of the S/G2 checkpoint may be due to inhibition of Chk1 kinase by UCN-01. The enhanced cytotoxicity produced when UCN-01 was combined with ara-C suggested a rationale for the use of this drug combination for tumors that might be susceptible to cell cycle checkpoint abrogation.

INTRODUCTION

Cell cycle checkpoints prevent premature initiation of cell cycle events and allow time for repair of DNA damage prior to replication or mitosis[1,2]. Many anticancer drugs induce DNA damage and activate cell cycle checkpoints. Abrogation of cell cycle checkpoints tends to sensitize cells to DNA damaging agents[3-8].

7-Hydroxystaurosporine (UCN-01) was initially described as a protein kinase C inhibitor[9,10]. UCN-01 inhibits the growth of human and murine tumor cell lines *in vitro* and exhibits antitumor activity in animal models[9,11]. At lower dose, UCN-01 has been reported to enhance cell killing by ionizing radiation and to synergize with cisplatin to preferentially kill cells with defective p53 function[3,4]. This enhancement has been related to an abrogation of the G2 checkpoint and activation of Cdc2 kinase. Bunch and Eastman[6] reported that UCN-01 abrogated the G2 arrest induced by cisplatin and enhanced cisplatin-induced cytotoxicity in CHO

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cells. UCN-01 also been shown to abrogate the S phase arrest and to potentiate the cytotoxicity of cisplatin or camptothecin[5,7].

As an inhibitor of DNA synthesis, arabinosylcytosine (ara-C) has the greatest cytotoxic effects during the S phase of cell cycle. The duration of exposure of cells to ara-C is directly correlated with cell kill because the longer exposure period allows ara-C to be incorporated into the DNA of a higher percentage of cells as they pass through S phase. The present study investigated the influence of UCN-01 on cell responses to ara-C in a p53-mutant background.

MATERIALS AND METHODS

Drugs, chemicals, and antibodies UCN-01 was provided by the Drug Synthesis Chemistry Branch, NCI. Aliquots were stored frozen at 10 mmol/L in dimethylsulfoxide, and further diluted in water immediately prior to each experiment. GST-Cdc25C (residues 200 through 256 fused to GST) was prepared as described previously[12]. Other drugs and reagents, unless otherwise mentioned, were purchased from Sigma.

Anti-cyclin A, B1, D1, Cdc2, Cdc25C monoclonal antibodies and anti-Chk1 polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-cyclin E and Cdk2 antibodies were purchased from PharMingen (San Diego, CA). [^{32}P]ATP, [^{14}C]thymidine, and [*methyl*-^{3}H]thymidine were purchased from New England Nuclear (Boston, MA).

Cell culture Human colon carcinoma HT29 cells were grown at 37 ºC in the presence of 5 % CO_2 in RPMI-1640 medium supplemented with 5 % fetal bovine serum (GIBCO-BRL), 2 mmol/L glutamine, 100 kU/L benzyl penicillin and 100 mg/L streptomycin.

Clonogenic assays Cells were treated with ara-C to be tested in association with UCN-01 for 8 h. Drugs were removed by rinsing the cultures once in drug-free medium, and UCN-01 was added for the next 16 h. Cells were then washed in drug-free medium and trypsinized. Two hundred and fifty cells were seeded in triplicate in T-25 tissue culture flasks. Colonies were grown for 2 weeks, then washed with phosphate-buffered saline (PBS), fixed with methanol and stained with methylene blue (0.04 %)[5]. Cloning efficiency of untreated cells was 78 %.

Flow cytometry Briefly, cells were harvested and fixed in 70 % ethanol. Before analysis by flow cytometry, cells were washed with PBS, treated with 8 g/L RNase and stained with 50 mg/L of propidium iodide for at least 30 min. DNA content was determined by FACScan flow cytometry (Becton Dickinson Immmmcytometry System).

DNA synthesis assays Briefly, cells were prelabeled with 1.85×10^5 Bq/L of [^{14}C]thymidine for 48 h. The rate of DNA synthesis was measured by 10 min pulses with 3.7×10^7 Bq/L of [*methyl*-^{3}H]thymidine. [^{3}H]incorporation was stopped by washing cells twice in ice-cold HBSS (Hanks' balanced salt solution), and then by scraping cells into 4 mL of ice-cold HBSS. One mL aliquots triplicate were then precipitated after addition of 100 μL of trichloroacetic acid. Samples were vortexed and centrifuged at 12 000×*g* at 4 ºC. The precipitates were then dissolved overnight at 37 ºC in 0.5 mL of 0.4 mol/L NaOH. Samples were counted by dual label liquid scintillation and [^{3}H]-values were normalized using [^{14}C]-counts.

Immunoblotting Cells were pelleted, washed once in PBS, and lysed at 4 ºC. Protein detection was performed using a protein assay kit according to the manufacturer's instructions (Bio-rad). Samples were separated by SDS-PAGE and electrophoretically transferred to Immobilon membranes (Millipore, Bedford, MA). Membranes were blocked overnight in PBS containing 0.1 % Tween-20 and 5 % nonfat dried milk, probed for 1 h with primary antibody and for 1 h with secondary antibody, and visualized by enhanced chemiluminescence. Representative data from an individual experiment shown in Fig 4 were reproducible at least twice.

Cdk and Chk1 kinase assays Cells were washed once in cold PBS, and lysed on ice as described previously[5]. Cell lysates (500 mg of total cell proteins per sample) were immunoprecipitated with anti-Cdk2, Cdc2 or Chk1 antibodies. For Cdk kinase assay, immune complexes were resuspended in kinase buffer (20 mmol/L Tris-HCl, 10 mmol/L $MgCl_2$, pH 7.5 containing 5 μmol/L unlabeled ATP and 3.7×10^5 Bq [^{32}P]ATP, with 3 μg of histone H1), and incubated at 37 ºC for 20 min. For Chk1 kinase assay, immune complexes were resuspended in reaction buffer (50 mmol/L Tris-HCl, pH 7.5, 10 mmol/L $MgC1_2$, 1 mmol/L DTT, 10 μmol/L ATP and 3.7×10^5 Bq [^{32}P]ATP, with 2 μg of the GST-Cdc25C), and incubated at 30 ºC for 30 min. Reactions were stopped by adding loading buffer and boiling samples for 5 min. Samples were loaded onto SDS-PAGE and electrophoresed at 120 V for 2 h. For quantitation of cyclin/Cdk kinase activity of

immunoprecipitates, gels were dried, and histone H1 phosphorylation was measured using a phosphorImager (Molecular Dynamics). Representative data from an individual experiment shown in Fig 5 were reproducible at least twice.

RESULTS

UCN-01 potentiated the cytotoxicity of ara-C Because previous studies indicated that UCN-01 potentiated the cytotoxicity of ionizing radiation, cisplatin and camptohecin, and abrogated cell cycle checkpoints[4,5,7], we tested whether UCN-01 would potentiate the cytotoxicity of ara-C. Ara-C is DNA synthesis inhibitor that arrest cells in S and G2 phases. Human colon carcinoma HT29 cells that are mutated for p53[13] were treated with ara-C in the presence or absence of UCN-01. Clonogenic assay showed that UCN-01 0.1 μmol/L markedly potentiated the cytotoxicity of ara-C (<1 μmol/L) (Fig 1), while UCN-01 or ara-C alone was not cytotoxic. The IC_{50} values of UCN-01 and ara-C were 28.1 and 15.3 μmol/L, respectively. The results indicated that UCN-01 potentiated the cytotoxicity of the DNA synthesis inhibitor.

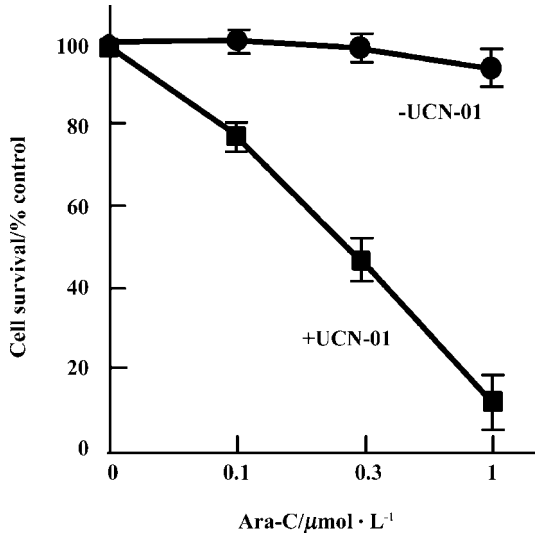

Fig 1. UCN-01 potentiates the cytotoxicity of ara-C. HT29 cells were treated with ara-C at the indicated concentrations for 8 h, after which cells were washed in drug-free medium and incubated with or without 0.1 μmol/L UCN-01 for 16 h. Ce11 survival was determined by clonogenic assays. *n*=3. Mean±SD.

UCN-01 antagonized the S/G2-phase arrest induced by ara-C The above result raised the question whether the potentiation of cytotoxicity of ara-C by UCN-01 might be associated with abrogation of the drug-induced cell cylce arrest. Flow cytometry analysis showed that UCN-01 reduced the S/G2-phase accumulation induced by ara-C, and decreased S phase population from 64.3 % to 29.1 % and G2 phase population from 18.9 % to 8.2 % (Fig 2). To determine whether the effect of UCN-0l was due to an inhibition of ara-C-induced S/G2-phase arrest or to G1 phase arrest[14], experiments were carried out in the presence of the mitosis inhibitor nocodazole to follow cell cycle pro-

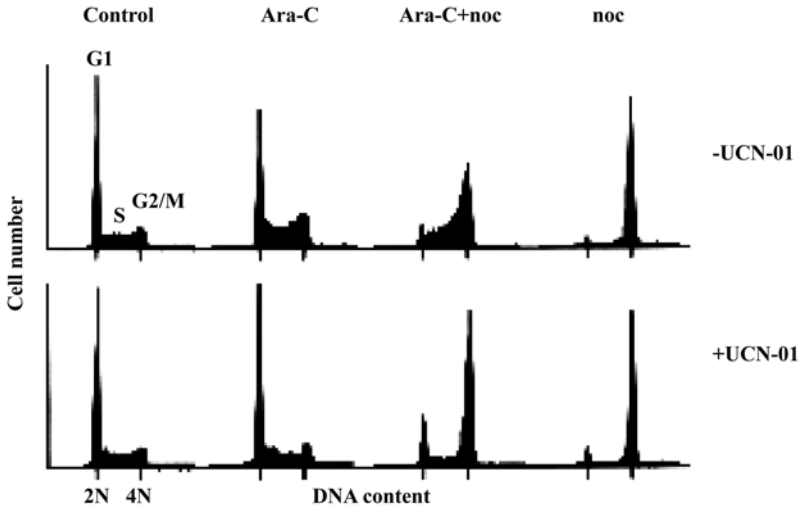

Fig 2. UCN-01 reduces the ara-C-induced slowing of progress through S-phase. H29 ce11s were treated with 1 μmol/L ara-C for 8 h. Following ara-C treatment, cells were washed in drug-free medium and treated with or without 0.1 μmol/L UCN-01 in the absense or presense of 0.4 mg/L nocodazole for 16 h. Cells were harvested and analyzed for cell cycle distribution by flow cytometry.

gression in the presence or absence of UCN-01 after ara-C treatment. Fig 2 showed that ara-C caused S/G2-phase accumulation both in the absence or presence of nocodazole, indicating an S/G2-phase arrest in the first cell cycle following ara-C treatment. Combined treatment of ara-C-treated cells with UCN-01 and nocodazole resulted in a large fraction of the cells being arrested in M phase. This indicated that cells were able to exit G1 and traverse S-phase unimpeded. UCN-01 alone did not significantly affect cell cycle progression.

UCN-01 accelerated DNA synthesis recovery in ara-C-treated cells We next investigated the effects of UCN-01 on the recovery of DNA synthesis following an 8-h treatment with ara-C. UCN-01 was added at the time of removal of ara-C. [^{3}H]thymidine pulses were performed at various times after ara-C treatment in the presence or absence of UCN-01. As expected, DNA synthesis was strongly suppressed by ara-C (approximately 90 %). This inhibition was only slowly reversible after removal of ara-C. UCN-01 accelerated the restoration of the ara-C-induced inhibition of DNA synthesis (Fig 3). Together, the results of Fig 2 and 3 indicated that UCN-01 antagonized ara-C-induced DNA synthesis inhibition and S/G2 phase delay.

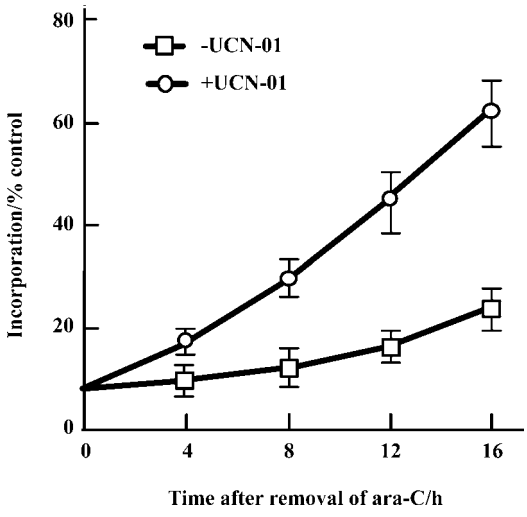

Fig 3. Effect of UCN-01 on DNA synthesis reactivation after ara-C- treatment. Cells were treated as described in Fig 1 (1 μmol/L ara-C) and harvested at the indicated times. [^{3}H]thymidine incorporation into DNA was measured by 10 min pulse- labeling. *n*=3. Mean±SD.

Alteration of cyclin levels associated with ara-C treatment in the absence and presence of UCN-01 To examine whether the cell cycle effects of ara-C and UCN-01 were associated with changes of cyclin/Cdk protein levels and activities, Western blotting and kinase assays were performed. Fig 4 showed that cyclin A and B1 proteins were increased in ara-C-treated cells and that UCN-01 partially prevented the increase of cyclins A and B1 induced by ara-C. Neither ara-C nor UCN-01 by themselves or in association affected Cdk 2 protein levels.

Activation of Cdc2 kinase by UCN-01 in ara-C-treated cells was associated with Cdc2 dephosphorylation and Cdc25C activation Cdc2 kinase is regulated and inhibited by hyperphosphorylation on tyrosine 15 and threonine-14[15]. The inactive phosphorylated form (on threonine-14 and tyrosine-15) migrates more slowly than dephosphorylated Cdc2 or the active form of Cdc2 (phosphorylated on threonine-161)[7]. Fig 4 showed that Cdc2 was hyperphosphorylated after ara-C treatment and that UCN-01 reduced ara-C-induced Cdc2 phosphorylation.

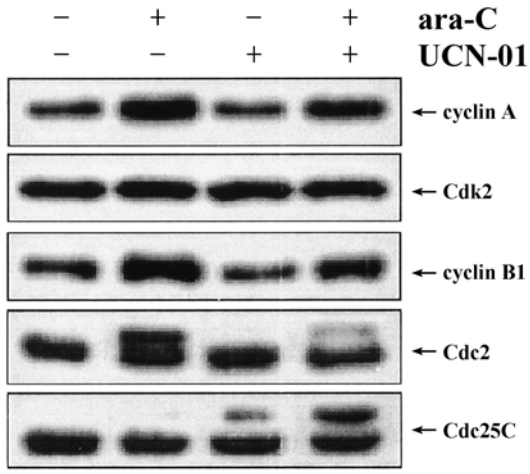

Fig 4. Western blot analysis of cyclins, Cdks and Cdc25C in cells treated with ara-C and/or UCN-01. Cells were treated as described in Fig 1 and cell lysates were prepared 16 h after ara-C (1 μmol/L) removal. The slow migrating Cdc2 and Cdc25C bands correspond to the inactive form of Cdc2 phosphorylated on threonine-14 and tyrosine-15, and the active hyperphosphorylated form of Cdc25C, respectively.

The Cdc2 inhibitory phosphorylation on threonine-14 and tyrosine-15 can be removed by the dual specific phosphatase, Cdc25C[15]. Cdc25C is also regulated by phosphorylation. In interphase cells Cdc25C is hypophosphorylated and inactive. As Cdc25C becomes hyperphosphorylated at the G2/M transition, its activity increases[15]. We assayed Cdc25C by Western blotting. The active form of Cdc25C migrates more slowly than the unphosphorylated inactive form of Cdc25C[16]. No

detectable hyperphosphorylation of Cdc25C occurred in ara-C treated cells. By contrast, UCN-01 treatment resulted in Cdc25C hyperphosphorylation in the absence or presence of ara-C (Fig 4). Thus, activation of Cdc25C is consistent with Cdc2 activation in UCN-01-treated cells.

Ara-C and UCN-01 changed cyclin/Cdk kinase activities Because cyclin levels regulate Cdk activities, we measured Cdc2 and Cdk2 activities after immunoprecipitation. Cdk2 kinase activity was increased in cells treated with ara-C but was within control values after UCN-01 treatment. By contrast, UCN-01 increased Cdc2 kinase activity in ara-C treated cells (Fig 5). These results indicate that the S/G2-phase delay induced by ara-C is not related to an inhibition of cyclin A/Cdk2 kinase activity.

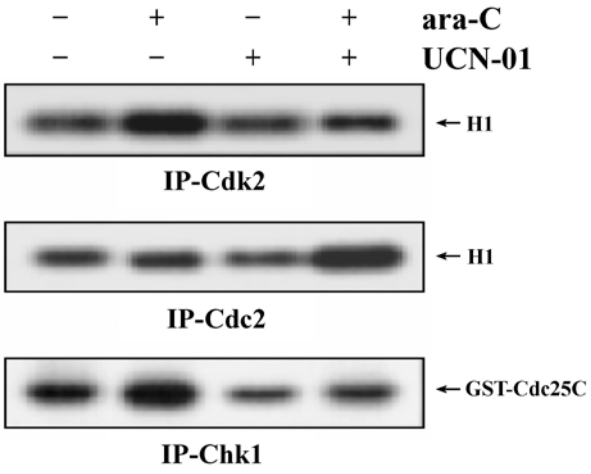

Fig 5. Effects of ara-C and UCN-01 on cyclins/Cdks and Chk1 kinase activities. Cells were treated as described in Fig 1 and kinase activities were measured 16 h after ara-C (1 μmol/L) removal. Immunoprecipitates were collected from cell extracts with anti-Cdc2 or anti-Cdk2 antibody, respectively. The kinase activity was measured using a PhosphorImager.

UCN-01 inhibited Chk1 protein kinase activation in ara-C-treated cells It has been shown that Cdc25C was negatively regulated by phosphorylation at serine-216 by the Chk1 kinase (see Fig 4). This phosphorylation induces Cdc25C binding to 14-3-3 protein and inactivation[11,17,18]. To test Chk1 activity in HT29 cells, immunoprecipitation was performed with anti-Chk1 polyclonal antibody and kinase activity was measured using a polypeptide containing the GST-Cdc25C (residues 200 through 256 fused to GST). Fig 5 showed that Chk1 activity was increased in ara-C-treated cells and that this activation was reduced in UCN-01-treated cells.

DISCUSSION

UCN-01 is a potent abrogator of the S and G2 checkpoints and potentiates the cytotoxicity of camptothecin, cisplatin and ionizing radiation with greatest efficiency in p53-mutant cells[4,5,7]. Our first goal was to determine whether this effect of UCN-01 was specific for anticancer agent ara-C. Our results indicated that UCN-01 was effective in association with ara-C and that UCN-01 had less or no effect when it was combined with the M-phase specific microtubule inhibitors (data not shown). This observation suggests that UCN-01 can interfere with cellular pathways associated with replication alteration[8].

UCN-01 markedly reduced S/G2-phase accumulation in ara-C-treated cells. The experiments in cells treated with nocodazole with UCN-01 and/or ara-C (Fig 2) demonstrated that UCN-01 exerted this effect by decreasing ara-C-induced S-phase arrest. This result suggests that S/G2-phase delay in ara-C-treated cells is an active cellular process possibly related to an S-phase checkpoint.

Consistent with this possibility, we found that the persistent DNA synthesis inhibition after an 8-h exposure to ara-C was reversed by UCN-01. These data suggest that cell cycle progression through S/G2- phase is regulated by checkpoints designed to slow cell cycle progression and allow time for recovery from perturbations such as ara-C. We propose that UCN-01 blocks this S/G2-phase checkpoint and as a result enhances cell death induced by ara-C.

Cyclin A/Cdk2 is essential for S-phase progression. Cyclin A binds to and acts as a positive regulator for both Cdk2 and Cdc2. Cyclin A increases during S-phase and drops at the end of the G2-phase. Our data show that ara-C can induce S/G2 arrest in spite of high cyclin A levels and the presence of active Cdk2. Abrogation of the ara-C-induced S/G2 arrest by UCN-01 was associated with a decrease of cyclin A/Cdk2 kinase activity. It is possible that the reduction of cyclin A/Cdk2 activity in UCN-01-treated cells is secondary to cells having progressed out of S-phase.

Formation and activation of cyclin B/Cdc2 complexes are required for mitotic entry[15] and DNA damage-induced G2 arrest is associated with Cdc2 kinase inactivation. Phosphorylation of Cdc2 on both threonine-14 and tyrosine-15 contributes to this inacti-

vation[15]. Our data indicate that Cdc2 is hyperphosphorylated and inactive in ara-C-treated cells (Fig 3). We also found that UCN-01 markedly enhanced the kinase activity of Cdc2 in ara-C-treated cells, perhaps because more cells were entering mitosis. It was interesting to note that Duneker *et al*[19] reported that cyclin B/Cdc2 kinase could stimulate semiconservative plasmid replication in yeast nuclear extracts through both a modification of the origin-bound complex and stimulation of elongation events. Thus, it is possible that activation of cyclin B1/Cdc2 by UCN-01 is involved in abrogating the S/G2 checkpoint elicited by ara-C.

Our data suggested that activation of Cdc2 by UCN-01 was related to activation of the dual specificity phosphatase, Cdc25C, that removed the two inhibitory phosphates on threonine-14 and tyrosine-15[20]. Graves and his colleague[21] reported that Cdc25C could be regulated by the Chk1 kinase. Chk1 is a key element of the DNA damage-induced S and G2 checkpoint[22-24]. Chk1 is activated in response to DNA damage and phosphorylates Cdc25C on serine-216. Cdc25C phosphorylation on serine-216 then promotes the formation of a complex between Cdc25C and 14-3-3 protein that results in Cdc25C inactivation[15,20]. Fig 5 showed that ara-C enhanced Chk1 kinase activity and that UCN-01 treatment prevented Chk1 activation. Thus, we propose that DNA replication altertions induced by are-C activate Chk1 kinase guarding against mitotic entry from S- and G2-phase. By inhibiting Chk1, UCN-01 blocks Cdc25C inactivation. Cdc2 can then be activated by dephosphorylation on threonine-14 and tyrosine-15. Our data indicate that Cdc2 kinase is negatively regulated by Chk1 in ara-C-treated cells and that Chk1 inhibition is involved in the abrogation of the S/G2 checkpoints.

The model for the ara-C-induced checkpoint signaling and modulation by UCN-01 is described in Fig 6: 1) ara-C-induced replication alterations activate Chk1 kinase, which in turns inhibits Cdc25C, and Cdc2 remains in its inactive form; 2) UCN-01 inhibits Chk1 kinase and prevents checkpoint activation. The S/G2 checkpoint target of UCN-01 appears to be Chk1. Considering the remarkable synergy conferred by UCN-01 in cells treated with ara-C, the observations have potential implications for the design of further clinical trials of Chk1 kinase inhibitor such as UCN-01 and drug combinations.

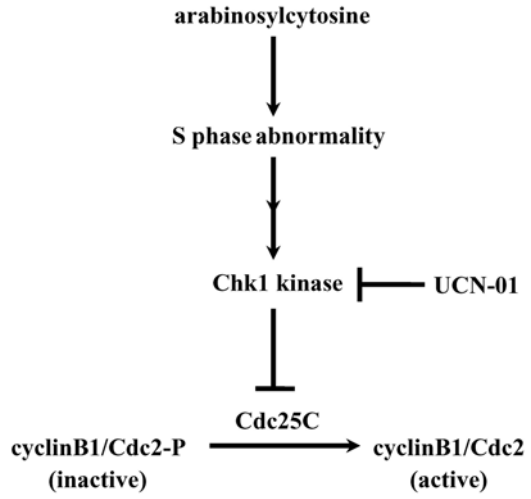

Fig 6. The model for the ara-C-induced checkpoint signaling and modulation by UCN-01.

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