

Dissecting the Roles of Checkpoint Kinase 1/CDC2 and Mitogen-Activated Protein Kinase Kinase 1/2/Extracellular Signal-Regulated Kinase 1/2 in Relation to 7-Hydroxystaurosporine-Induced Apoptosis in Human Multiple Myeloma Cells

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ABSTRACT

The functional roles of Cdc2 and checkpoint kinase 1 (Chk1) in synergistic interactions between 7-hydroxystaurosporine (UCN-01) and mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitors [e.g., 2-(2-chloro-4-iodophenylamino)-*N*-cyclopropylmethoxy-3,4-difluorobenzamide (PD184352)] were examined in human multiple myeloma cells in relation to MEK1/2/ERK1/2 activation and lethality. Time course studies revealed that MEK1/2/extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation preceded Cdc2 dephosphorylation (Tyr15) after UCN-01 exposure. Furthermore, enforced expression of Cdc2 or small inducible RNA (siRNA)-mediated Cdc2 knockdown failed to modify ERK1/2 activation status in either the presence or absence of UCN-01, arguing against a causal relationship between these events. However, ectopic expression of Cdc2 sensitized cells to the lethality of UCN-01/MEK inhibitor regimen, whereas Cdc2 knockdown by siRNA signifi-

cantly diminished the lethal effects of this combination. Conversely, Chk1 knockdown by siRNA enhanced lethality mediated by UCN-01/PD184352. It is interesting that Chk1 knockdown reduced basal ERK1/2 activation and antagonized the ability of UCN-01 to activate ERK1/2. Finally, ectopic expression of constitutively active MEK1 significantly protected cells from the UCN-01/MEK1/2 inhibitor regimen without modifying Cdc2 activation status. Together, these findings indicate that although UCN-01-mediated Chk1 inhibition and Cdc2 activation are unlikely to be responsible for MEK1/2/ERK1/2 activation, both of these events contribute functionally to enhanced lethality in cells coexposed to MEK inhibitors. They also suggest a role for Chk1 in UCN-01-induced ERK1/2 activation, implying the existence of a heretofore unrecognized link between Chk1 and ERK1/2 signaling.

Cell cycle checkpoints provide mechanisms by which cells undergo arrest or delay at specific phases of the cell cycle in response to DNA damage and permit attempts at DNA re-

pair. Checkpoint regulation involves multiple complex mechanisms (Kastan and Bartek, 2004) in which Chk1 and Chk2 act as critical messengers transducing DNA damage signals to checkpoint “effectors,” including p53, Cdc25s, and Cdks (Niida and Nakanishi, 2006). Studies in Chk1-deficient mouse cells suggest an essential role for this protein in DNA damage and DNA replication checkpoint responses (Takai et al., 2000). Furthermore, Chk1 deficiency results in the premature onset of mitosis through dephosphorylation of Cdc2 at inhibitory tyrosine 15 residues and activation of Cdc2 (Niida and Nakanishi, 2006). In this context, Chk1 has been

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ABBREVIATIONS: Chk1, checkpoint kinase 1; MEK, mitogen-activated protein kinase kinase; UCN-01, 7-hydroxystaurosporine; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; RNAi, RNA interference; DMSO, dimethyl sulfoxide; PKC, protein kinase C; siRNA, small interference RNA; FITC, fluorescein isothiocyanate; PI, propidium iodide; PARP, poly(adenosine) diphosphate-ribose polymerase; ds, double-stranded; CA, constitutively active; 7-AAD, 7-amino-actinomycin D; HA, hemagglutinin; PD98059, 2'-amino-3'-methoxyflavone; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene; PD184352, 2-(2-chloro-4-iodophenylamino)-*N*-cyclopropylmethoxy-3,4-difluorobenzamide.

shown to phosphorylate/inactivate Cdc25s (e.g., Cdc25C and subsequently Cdc25A), resulting in the accumulation of tyrosine 15-phosphorylated Cdc2 (Niida and Nakanishi, 2006). The "inappropriate" activation of Cdc2 by itself promotes apoptosis (Shimizu et al., 1995).

The development of checkpoint defects represents a common characteristic of malignant versus normal cells and represents a potential target for therapeutic intervention (Kastan and Bartek, 2004). UCN-01 was originally designed as a protein kinase C inhibitor but was subsequently shown to inhibit, in addition to cyclin-dependent kinases, pyruvate dehydrogenase kinase 1 (Sato et al., 2002) and Chk1 (Graves et al., 2000). Inhibition of Chk1 by UCN-01 and consequently activation of Cdc25C (Kohn et al., 2002) and Cdc25A lead to abrogation of the G₂/M and/or S checkpoint, thus sensitizing tumor cells to DNA-damaging agents (Sampath et al., 2006) and radiation (Yu et al., 2002). In preclinical studies, UCN-01 induces apoptosis in human leukemia cells in a p53-independent manner when administered at concentrations in the submicromolar range (Shao et al., 1997; Byrd et al., 2001). Very recently, it was reported that UCN-01-mediated Chk1 inhibition increases DNA damage and triggers phosphorylation of ataxia telangiectasia mutated (ATM)-related protein (ATR) targets, including Chk1 itself (Syljuasen et al., 2005). Recent clinical studies have shown the feasibility of achieving free UCN-01 concentrations as high as 400 nM with a 3-h infusion (Hotte et al., 2006). However, it is currently unclear how UCN-01 induces apoptosis *in vitro* and inhibits tumor growth *in vivo*.

The mitogen-activated protein kinase (MAPK) family consist of four parallel modules [i.e., those associated with the extracellular signal regulating kinase pathways (ERK1/2 and ERK5), c-Jun N-terminal kinase, and p38 MAPK]. These signaling pathways are activated by diverse stimuli, including growth factors, inflammation, and environmental stresses, among others (Chang and Karin, 2001). Although exceptions exist, ERK1/2 and ERK5 activation is associated with cell proliferation and survival, whereas c-Jun N-terminal kinase and p38 MAPK activation is generally linked to the induction of cell death (Chang and Karin, 2001). Mechanisms by which ERK1/2 activation opposes cell death include inactivation of caspase 9 (Allan et al., 2003) or BAD (Scheid et al., 1999). In general, activation of the MEK1/2/ERK1/2 pathway is mediated by Ras/Raf signaling (Steelman et al., 2004). However, evidence has accumulated indicating that activation of MEK1/2/ERK1/2 may involve cross-talk between diverse signaling pathways (Steelman et al., 2004).

In previous studies, our group reported that exposure of human leukemia (Dai et al., 2001) and myeloma (Dai et al., 2002) cells to UCN-01 resulted in a marked activation of MEK1/2/ERK1/2. This phenomenon has also been observed in cell lines derived from human solid tumors including breast, prostate (McKinstry et al., 2002), head and neck squamous cancer (Facchinetti et al., 2004), and recently in an *in vivo* mouse tumor model independently of tumor p53 status (Hawkins et al., 2005). Furthermore, prevention of MEK1/2/ERK1/2 activation (e.g., by pharmacological MEK1/2 inhibitors) dramatically increases apoptosis. Because inhibition of Chk1 leads to activation of Cdc2 (Graves et al., 2000), and unscheduled activation of Cdc2 is a potent inducer of apoptosis (Shimizu et al., 1995), it was tempting to speculate that UCN-01-mediated MEK1/2/ERK1/2 activation

represented a compensatory response to inappropriate activation of Cdc2. However, the precise relationship between these events has not yet been investigated rigorously. To address these issues, we used genetic approaches to investigate the functional relationship between Chk1 inhibition/Cdc2 activation and MEK1/2/ERK1/2 activation in human multiple myeloma cells. Our results indicate that although Cdc2 dephosphorylation/activation is not responsible for MEK1/2/ERK1/2 activation in cells exposed to UCN-01, this phenomenon does contribute to the lethality of the UCN-01/MEK1/2 regimen. They also provide evidence of a functional role for down-regulation/inhibition of Chk1 and MEK1/2 in synergistic interactions between these agents. Finally, our results suggest the presence of a heretofore unrecognized relationship between Chk1 and MEK1/2/ERK1/2 activation in UCN-01-treated cells.

Materials and Methods

Cells and Reagents. The human multiple myeloma cell line U266 (mutant p53) was purchased from American Type Culture Collection (Manassas, VA). Cells were maintained in 10% fetal bovine serum/RPMI 1640 medium, as described previously (Dai et al., 2002). RPMI 8226 cells (mutant p53) were kindly provided by Alan Lichtenstein (University of California at Los Angeles, Los Angeles, CA) and cultured as reported previously (Zhang et al., 2003). All experiments were performed using logarithmically growing cells ($4-6 \times 10^5$ cells/ml). UCN-01 was kindly provided by the Cancer Treatment and Evaluation Program (National Cancer Institute, Rockville, MD), dissolved in dimethyl sulfoxide (DMSO) at a stock concentration of 1 mM, stored at -20°C , and subsequently diluted with serum-free RPMI medium before use. The selective MEK inhibitor PD184352 was purchased from Upstate Biotechnology (Lake Placid, NY). Other MEK inhibitors (PD98059 and U0126) were supplied by Calbiochem (San Diego, CA). The inhibitors were dissolved in sterile DMSO and stored frozen under light-protected conditions at -20°C . In all experiments, the final concentration of DMSO did not exceed 0.1%.

Retroviral Infection. Human wild-type Cdc2 cDNA was kindly provided by Dr. David Morgan (University of California at San Francisco, San Francisco, CA), released with BamHI, and subsequently cloned into a pLXIN retroviral vector. Procedures for transfection of PT67 packaging line and infection of RPMI 8226 cells to establish lines ectopically expressing wild-type Cdc2 have been described in detail previously (Hyun et al., 2000).

RNA Interference. RPMI 8226 and U266 cells were transfected with 10 μM dsRNAi oligonucleotides corresponding to either Cdc2 or Chk1, which were purchased from Cell Signaling Technology (Danvers, MA). Nontargeting control siRNA (nonspecific random sequence) was purchased as a solution from Dharmacon (Lafayette, CO). Transfections were performed by using the Amaxa Nucleofector device (program G-015 for RPMI 8226 and X-005 for U266) with Cell Line Nucleofector Kit V (for RPMI 8226) and Kit C (for U266) (Amaxa GmbH, Cologne, Germany) according to the manufacturer's instructions. After incubation at 37°C for 24 h, transfected cells were treated with UCN-01 \pm MEK1/2 inhibitors. At the indicated intervals, generally 24 or 48 h, cells were harvested and subjected to analysis of apoptosis and Western blot.

Transient and Stable Transfection with Constitutively Active MEK1. Hemagglutinin (HA)-tagged constitutively active (CA) MEK1 cDNA (activating mutations of serine 218 and 222 to aspartic acid) in a pUSEamp vector was purchased from Upstate Biotechnology (Lake Placid, NY). U266 and RPMI 8226 cells (2×10^7) were transfected with the CA MEK1 construct or its empty vector (pUSE), respectively, using the Amaxa Nucleofector as described under RNA Interference. After incubation at 37°C for 24 h, transfected U266

cells were treated with UCN-01 \pm MEK inhibitors for another 24 to 48 h and then subjected to analysis of apoptosis and Western blot. Transfected RPMI 8226 cells were continuously cultured under selection by G418 (750 μ g/ml) to establish stable lines with ectopic expression of CA MEK1.

Cyclin B1-Cdk1/Cdc2 Kinase Assay. Cdc2 kinase activity was detected using a Cdc2 Kinase Assay Kit (Upstate Biotechnology) according to the manufacturer's instructions. In brief, 2×10^7 cells were lysed by sonication in $1 \times$ cell lysis buffer containing 1 mM phenylmethylsulfonyl fluoride. Protein (400 μ g) per condition was incubated with or without (as immunoprecipitation control) 10 μ l of an anticyclin B1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 2 h at 4°C. Protein A/G-conjugated agarose beads (20 μ l) were then added and incubated overnight at 4°C. The beads were washed four times with cell lysis buffer and once with kinase buffer. The immunoprecipitates were incubated with assay dilution buffer containing 400 μ g/ml histone H1 (Upstate Biotechnology), 100 μ M ATP, 15 mM MgCl₂, and 2 μ Ci (0.074 MBq) [γ -³²P]ATP at 30°C for 20 min. The reaction was terminated by adding 3 \times SDS sample buffer and boiling for 5 min. γ -³²P-labeled histone H1 was separated by 12% SDS-polyacrylamide gel electrophoresis and visualized by exposure of the dried gels to X-ray film (Eastman Kodak, Rochester, NY) at -80°C for 1 h.

Western Blot Analysis. Western blot samples were prepared from whole-cell pellets using Triton X-100 buffer as described previously (Hyun et al., 2000). The amount of total protein was quantified using Coomassie Protein Assay Reagent (Pierce, Rockford, IL). Equal amounts of protein (20 μ g) were separated using a precast SDS-PAGE gel (Invitrogen, Carlsbad, CA) and electrotransferred onto nitrocellulose membranes. For detecting phosphorylated proteins, no SDS was included in the transfer buffer, and Tris-buffered saline was used throughout. Blots were probed with the appropriate dilution of primary antibodies as follows. Where indicated, the blots were reprobbed with antiactin (Sigma, St. Louis, MO) or antitubulin antibody (Oncogene Inc., San Diego, CA) to ensure equal loading and transfer of proteins. Primary antibodies included phospho-p44/42 (Thr202/Tyr204) MAPK (ERK) antibody, p44/42 MAPK antibody, MAP kinase 1/2 (MEK1/2) antibody, phospho-MEK1/2 antibody (all from Cell Signaling Technology), phospho-Cdc2 (Tyr15) and Cdc2 antibody (Santa Cruz Biotechnology), poly(adenosine) diphosphate-ribose polymerase (PARP) antibody (BIOMOL Research Laboratories, Plymouth Meeting, PA), cleaved PARP (89 kDa) antibody (Cell Signaling Technology), Chk1 antibody (Cell Signaling Technology), HA antibody (Santa Cruz Biotechnology), caspase-3 (BD Transduction Laboratories, Lexington, KY), and cleaved caspase-3 (17 kDa) (Cell Signaling).

Assessment of Apoptosis. The extent of apoptosis was evaluated by Annexin V-fluorescein isothiocyanate (FITC) staining and flow cytometry as described previously (Dai et al., 2002). In brief, 1×10^6 cells were stained with Annexin V-FITC (BD PharMingen, San Diego, CA) and 5 μ g/ml propidium iodide (PI; Sigma) in $1 \times$ binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaOH, and 2.5 mM CaCl₂) for 15 min at room temperature in the dark. Samples were then analyzed by flow cytometry within 1 h to determine the percentage of cells displaying Annexin V positivity. In some cases, cell death was also analyzed by 7-AAD staining and flow cytometry. In brief, cells were incubated with 0.5 μ g/ml 7-AAD at 37°C for 30 min and then subjected to flow cytometric analysis.

Statistical Analysis. For flow cytometric analyses of Annexin V/PI and 7-AAD, values represent the means \pm S.D. for at least three separate experiments performed in triplicate experiments. The significance of differences between experimental variables was determined using the Student's *t* test.

Results

The temporal relationship between Cdc2 and MEK1/2/ERK1/2 activation was first examined. After exposure of

RPMI 8226 cells to 150 nM UCN-01, Cdc2 activation, manifested by Tyr15 dephosphorylation, was discernible beginning at 18 h (Fig. 1A) and was more apparent by 24 h (data not shown). On the other hand, MEK1/2 and ERK1/2 phosphorylation/activation was noted as early as 3 h after UCN-01 exposure and increased over the ensuing 18 h. Similar results were observed in U266 cells (Fig. 1B). Thus, from a purely temporal standpoint, MEK1/2 and ERK1/2 phosphorylation preceded that of Cdc2 dephosphorylation after UCN-01 exposure and therefore seemed unlikely to represent a consequence of Cdc2 activation.

To test this possibility more rigorously, a genetic approach was used. To this end, RPMI 8226 cells were infected with a retrovirus encoding HA-tagged wild-type Cdc2. Clones were isolated displaying a slowly migrating HA-Cdc2 species compared with endogenous Cdc2 (Fig. 2A). Data from a representative clone (designated 8226/Cdc2-14) are shown in Fig. 2, A and B. A Cdc2 kinase assay revealed a clear increase in cyclin B1-Cdc2 activity in 8226/Cdc2-14 cells (Fig. 2A, bottom). However, despite the increase in Cdc2 expression and activity, basal levels of phospho-ERK1/2 were roughly equivalent in 8226/Cdc2-14 cells and 8226/neo controls (Fig. 2A). Results with an additional clone (designated 8226/Cdc2-1) yielded similar results (data not shown). These findings indicate that ectopic expression of Cdc2, accompanied by an increase in Cdc2 activity, does not by itself alter the status of ERK1/2 phosphorylation/activation.

Signaling responses of cells ectopically expressing Cdc2 to UCN-01 were then examined. It is noteworthy that 8226/Cdc2-14 cells were clearly susceptible to UCN-01-mediated Cdc2 tyrosine 15 dephosphorylation (including both endogenous and exogenous protein) (Fig. 2B), consistent with robust activation of Cdc2 after exposure to this Chk1 inhibitor (Wang et al., 1995; Dai et al., 2001). However, the relative increase in ERK1/2 phosphorylation/activation after UCN-01 exposure was roughly equivalent in the two cell lines. Taken together with the preceding findings, these observations argue against the possibility that the capacity of UCN-01 to

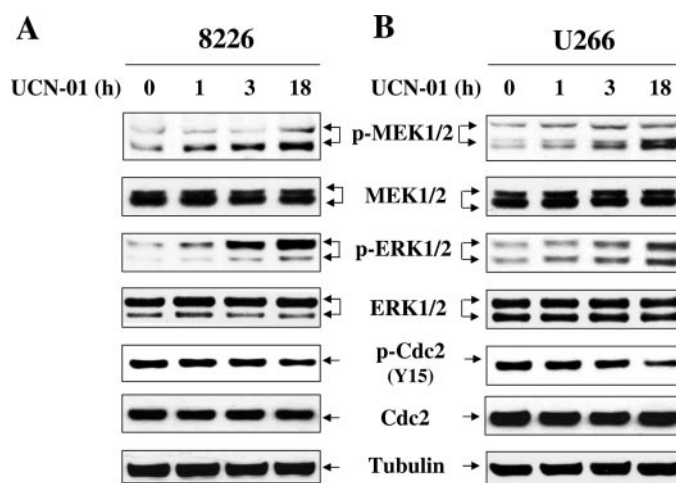


Fig. 1. The temporal relationship between MEK/ERK and Cdc2 activation in response to UCN-01. RPMI 8226 (A) and U266 (B) cells were exposed to 150 nM UCN-01. At the indicated intervals, the cells were harvested and subjected to Western blot analysis using the designated primary antibodies. Each lane was loaded with 20 μ g of protein; the blots were subsequently stripped and reprobbed with antitubulin antibody to ensure equal loading and transfer. Two additional studies yielded equivalent results.

trigger ERK1/2 activation in multiple myeloma cells represents a consequence of UCN-01-mediated Cdc2 activation.

The impact of increased expression and activity of Cdc2 on responses to UCN-01 and the MEK1/2 inhibitor PD184352, alone and in combination, was then investigated. Consistent with our previous reports (Dai et al., 2002), coadministration of PD184352, even at low concentrations (e.g., 2 μ M), significantly increased the lethality of UCN-01 in myeloma cells (Fig. 3A). Furthermore, in RPMI 8226 cells stably expressing ectopic Cdc2 (e.g., 8226/Cdc2-14), treatment with 150 nM UCN-01 alone for 24 h resulted in a modest but statistically significant increase in apoptosis, reflected by Annexin V positivity, compared with empty-vector controls ($P < 0.05$ in each case; Fig. 3A). Moreover, an even greater increase in the lethality of the PD184352/UCN-01 combination regimen was observed in these cells ($P < 0.02$ compared with values for 8226/Neo controls). Similar results were obtained in the 8226/Cdc2-1 clone, or when another MEK1/2 inhibitor, U0126 (20 μ M), was used (data not shown). Western blot analysis confirmed a modest but discernible increase in caspase-3 cleavage and PARP degradation in 8226/Cdc2-14 cells exposed to UCN-01 alone and in combination with PD184352 (Fig. 3B). Taken in conjunction with the previous findings, these results suggest that whereas activation of Cdc2 is unlikely to be responsible for UCN-01-mediated ERK1/2 activation, increased Cdc2 activity does contribute functionally to the lethality of UCN-01 when this agent is combined with an MEK1/2 inhibitor.

To determine whether knockdown of Cdc2 expression would reciprocally affect responses to the PD184352/UCN-01 regimen, an siRNA approach was used. Transient transfection of RPMI 8226 cells with siRNA oligonucleotide corresponding to Cdc2 resulted in a clear decrease in the expres-

sion of Cdc2 compared with cells transfected with control siRNA (Fig. 4A). It is noteworthy that there was no discernible change in levels of phosphorylated/activated ERK1/2 after down-regulation of Cdc2 by siRNA. Identical results were observed in U266 cells in which Cdc2 expression was down-regulated by the same siRNA approach (Fig. 4B). These findings are in accord with the observation that ectopic expression and increased activity of Cdc2 failed to modify ERK1/2 activation status (Fig. 2A). However, knockdown of Cdc2 by siRNA modestly but significantly reduced the lethal effects of the PD184352/UCN-01 regimen ($P < 0.02$ versus control siRNA; Fig. 4C). Knockdown of Cdc2 by siRNA also significantly reduced UCN-01/PD184352-mediated lethality in U266 cells ($P < 0.05$; data not shown). Together, these findings provide further support for the notion that activation of Cdc2 contributes to apoptosis induction by this drug regimen, but is not responsible for ERK1/2 activation in UCN-01-treated cells.

In light of the preceding evidence that activation of Cdc2 was not responsible for ERK1/2 activation induced by UCN-01, the possibility that the latter event might be related to effects of UCN-01 on Chk1 was investigated. To this end, RPMI 8226 cells were transfected with a Chk1 siRNA oligonucleotide, and Western blot analysis revealed marked down-regulation of Chk1 expression after 24 h (Fig. 5A). As shown in Fig. 5B, knockdown of Chk1 by siRNA failed to increase levels of phospho-ERK1/2; in fact, basal levels of phospho-ERK1/2 were, if anything, slightly reduced compared with cells transfected with control siRNA. UCN-01 robustly induced ERK1/2 activation in cells transfected with control siRNA, as noted in untransfected cells (Fig. 1A); however, Chk1 knockdown by siRNA markedly diminished the ability of UCN-01 to induce ERK1/2 (Fig. 5B). Similar

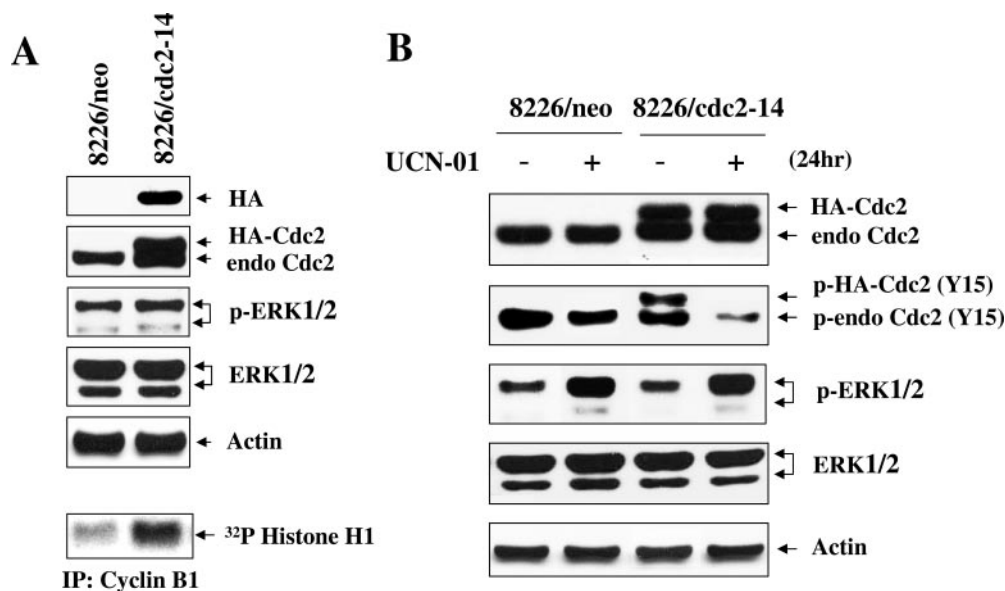


Fig. 2. Ectopic expression of wild-type Cdc2 does not affect basal or UCN-01-mediated ERK1/2 phosphorylation. A, RPMI 8226 were infected with a retrovirus encoding HA-tagged human wild-type Cdc2 or its empty-vector counterpart (pLXIN), and stable clones were obtained through G418 selection. Untreated 8226/neo and 8226/cdc2-14 cells were lysed and analyzed by Western blot using the indicated primary antibodies to monitor ectopic expression of HA-cdc2 and effects on phosphorylation of ERK1/2. Alternatively, a cyclin B1/Cdc2 kinase activity assay was performed in 8226/neo and 8226/Cdc2-14 cells as described under *Materials and Methods*. Cdc2 kinase activity is reflected by incorporation of [³²P]ATP into histone H1. B, 8226/Cdc2-14 and 8226/neo cells were exposed to 150 nM UCN-01 for 24 h, after which cells were lysed and subjected to Western blot analysis using the indicated primary antibodies. Each lane was loaded with 20 μ g of protein; blots were stripped and reprobed with antiactin antibodies to ensure equal loading and transfer. The results derived from a representative clone (clone 14) are shown. Studies used two additional clones yielded equivalent results.

results were obtained in U266 cells in which Chk1 was down-regulated by siRNA (data not shown). These findings raise the possibility that the presence of Chk1 may be required for UCN-01-induced ERK1/2 activation and indicate that knockdown of Chk1 by itself slightly reduces rather than increases ERK phosphorylation.

Parallel studies were performed to determine what effect, if any, knockdown of Chk1 would have on the response of cells to UCN-01 and PD184352, alone and in combination. As shown in Fig. 5C, RPMI 8226 cells transfected with Chk1 siRNA were significantly more sensitive than controls to apoptosis induced by PD184352 (1 μ M) and UCN-01 (150 nM), alone and particularly in combination ($P < 0.05$ – 0.002 in each case). Similar results were obtained when the MEK1/2 inhibitor U0126 was used (data not shown). Therefore, taken in conjunction with the preceding observations, these findings suggest that down-regulation/inactivation of Chk1 contributes to the enhanced lethality of the PD184352/UCN-01 regimen but, like Cdc2 activation, is not responsible for UCN-01-mediated ERK1/2 activation. In fact, these findings suggest that Chk1 knockdown, if anything, diminishes the ability of UCN-01 to induce ERK1/2 activation.

Attempts were then made to determine whether activation of MEK1/2/ERK1/2 could affect Cdc2 activation and protect cells from exposure to UCN-01 \pm MEK1/2 inhibitor. To this

end, U266 cells were transiently transfected with a construct encoding an HA-tagged CA MEK1 protein or its empty vector, after which they were exposed to UCN-01 (100 nM) + 50 μ M PD98059. The MEK1/2 inhibitor PD98059 was used in these studies instead of PD184352 in view of evidence that it is unable to block the actions of CA MEK1 (Racke et al., 1997). As shown in Fig. 6A, U266 cells transfected with the activated mutant MEK1 construct displayed a clear increase in expression of HA-tagged MEK1 and a marked increase in phosphorylation/activation of ERK1/2. However, levels of phospho-Cdc2 (tyrosine 15) were equivalent in cells transfected with empty vector and CA MEK1. It is noteworthy that enforced activation of ERK1/2 very significantly attenuated the lethal effects of the UCN-01/PD98059 regimen ($P < 0.002$ for 24-h treatment and <0.02 for 48-h treatment, respectively; Fig. 6B). Enforced activation of ERK1/2 by CA MEK1 provided comparable protection in cells in which Chk1 was knocked down by siRNA (data not shown). Together, these findings indicate that enforced activation of MEK1/2/ERK1/2 protects myeloma cells from the UCN-01/MEK1/2 inhibitor combination without altering the status of Cdc2 activation. They also imply that inactivation of the MEK1/2/ERK1/2 cascade plays a significant functional role, independent of Cdc2 activation, in the lethality of this regimen.

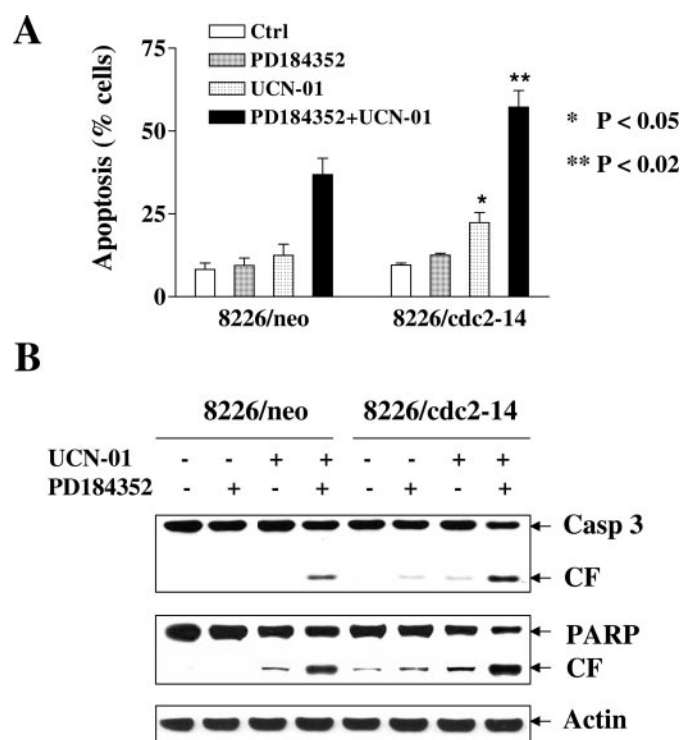


Fig. 3. Enforced activation of Cdc2 sensitizes RPMI 8226 to the lethality of the PD184352/UCN-01 regimen. **A**, 8226/Cdc2-14 cells and 8226/neo cells were incubated with 150 nM UCN-01 \pm 2 μ M PD184352 for 24 h, after which the percentage of apoptotic cells was determined by Annexin V-FITC/PI staining and flow cytometry. Results represent the means \pm S.D. for three separate experiments performed in triplicate. *, significantly greater than the value for empty vector controls with same treatment (*, $P < 0.05$; **, $P < 0.02$). **B**, 8226/Cdc2-14 cells were treated as described in **A**, after which cells were lysed and subjected to Western blot analysis to assess cleavage of caspase-3 and PARP. CF, cleavage fragment. Each lane was loaded with 20 μ g of protein; blots were stripped and reprobed with antiactin antibodies to ensure equal loading and transfer. Two additional studies yielded equivalent results.

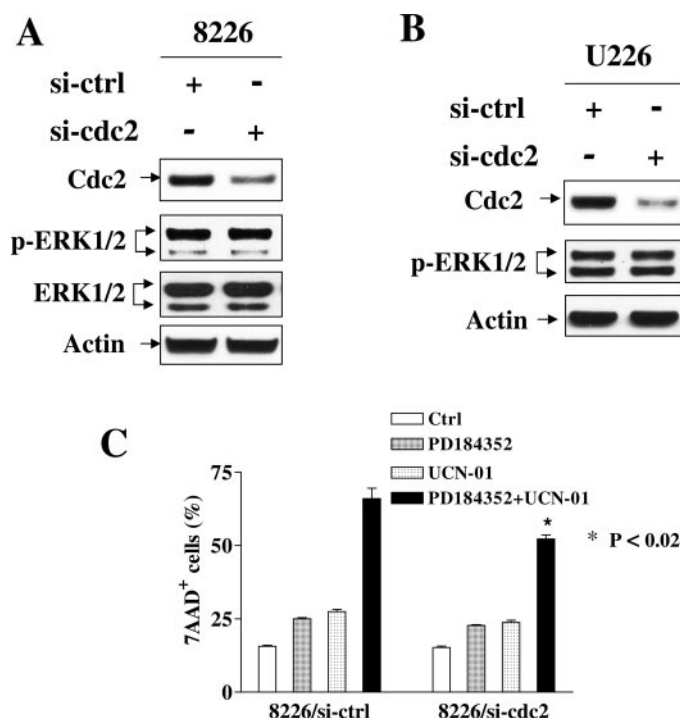


Fig. 4. Knockdown of Cdc2 by siRNA does not affect ERK1/2 activation but blunts lethality of the PD184352/UCN-01 regimen. RPMI 8226 (**A**) and U266 (**B**) cells were transiently transfected with ds RNAi oligonucleotides directed against Cdc2 or nontargeting siRNA control oligonucleotides, respectively, and then incubated at 37°C for 24 h, after which cells were lysed and subjected to Western blot analysis using the indicated primary antibodies. Each lane was loaded with 20 μ g of protein; blots were stripped and reprobed with antiactin antibodies to ensure equal loading and transfer. Two additional studies yielded equivalent results. **C**, RPMI 8226 cells transfected with Cdc2 siRNA or control nontargeting siRNA were treated for 24 h with 150 nM UCN-01 \pm 2 μ M PD184352, after which the percentage of apoptotic cells was determined by 7-AAD uptake as described under *Materials and Methods*. Results represent the means \pm S.D. for three separate experiments performed in triplicate. *, significantly lower than the values for cells treated with control nontargeting siRNA ($P < 0.02$).

Finally, parallel studies were carried out in RPMI 8226 cells stably expressing an HA-tagged CA MEK1 using a clone designated 8226/CA-MEK1-20 (Fig. 6C, inset). Consistent with results involving U266 cells transiently transfected with CA MEK1, 8226/CA-MEK1-20 cells were significantly less sensitive to apoptosis induced by combined exposure to 100 nM UCN-01 + 50 μ M PD98059 than their empty-vector counterparts ($P < 0.005$; Fig. 6C). These results were confirmed when cleavage of PARP and caspase 3 was monitored by Western blot analysis (Fig. 6D). Western blot analysis also revealed that ectopic expression of CA MEK1 enforced ERK1/2 phosphorylation/activation (Fig. 6D) without affecting Tyr15 dephosphorylation status of Cdc2 (data not shown), as noted in the case of U266 cells transiently transfected with CA MEK1 (Fig. 6A). It is noteworthy that UCN-01 exposure resulted in an even greater activation of ERK1/2 than in cells transfected with empty vector (Fig. 6D). In addition, coadministration of PD98059 was unable to block CA MEK1-driven phosphorylation/activation of ERK1/2 (Racke et al., 1997), in either the absence or presence of UCN-01. In contrast, activation of endogenous ERK1/2 in both empty vector- and CA MEK1-transfected cells was substantially reduced by this MEK1/2 inhibitor. Together, these observations support the notion that attenuation of UCN-01-mediated MEK1/2/ERK1/2 activation by MEK1/2 inhibitors, despite failing to modify Cdc2 activation status, contributes functionally to the lethality of this regimen.

Discussion

Our group has reported that exposure of diverse hematopoietic (e.g., leukemia and myeloma) and nonhematopoietic malignant cells to UCN-01 resulted in Chk1 inhibition and a striking induction of the MEK1/2/ERK1/2 cascade. Pharmacological interruption of the former event triggered a pronounced apoptotic response, accompanied by increased Cdc2 dephosphorylation and activation (Dai et al., 2001, 2002;

McKinstry et al., 2002). These findings strongly support the notion that MEK1/2/ERK1/2 activation is a protective compensatory response in cells exposed to UCN-01 and plays a critical role in diminishing UCN-01-related lethality. They also raise the possibility of a functional relationship between these signaling pathways. However, the mechanism by which MEK1/2/ERK1/2 is activated in this setting and the issue of whether UCN-01-mediated Chk1 inhibition and/or Cdc2 activation contributes to this phenomenon remain to be elucidated. Based on existing knowledge, several plausible possibilities arise. For example, activation of MEK1/2/ERK1/2, which exerts antiapoptotic effects through multiple mechanisms (Steelman et al., 2004), has been implicated in the orderly progression of cells across the G₂M boundary and through mitosis (Cross and Smythe, 1998; Abbott and Holt, 1999). Second, unscheduled activation of Cdc2 is known to be a highly potent apoptotic stimulus (Shimizu et al., 1995). From an operational standpoint, it could be argued that activation of ERK1/2 serves as a compensatory process that keeps the proapoptotic actions of Cdc2 in check and permits mitosis to proceed in an orderly manner. A corollary of this concept is that unscheduled activation of Cdc2 may be defined, at least in part, as activation that occurs in the absence of MEK1/2/ERK1/2 induction. Thus, it was tempting to postulate that UCN-01, by inappropriately activating Cdc2 via Chk1 inhibition and the consequent sparing of Cdc25C (Graves et al., 2000; Dai et al., 2001), elicited a compensatory activation of the prosurvival MEK1/2/ERK1/2 pathway. However, the results of the present study indicate that although the lethality of UCN-01 and of the UCN-01/MEK1/2 inhibitor regimen is associated with Chk1 inactivation and Cdc2 activation, the latter events are unlikely to be responsible for MEK1/2/ERK1/2 activation. Instead, other yet-to-be-identified factors must be involved in this phenomenon.

The present results argue strongly that Cdc2 activation plays a functional role in UCN-01/MEK1/2 inhibitor-medi-

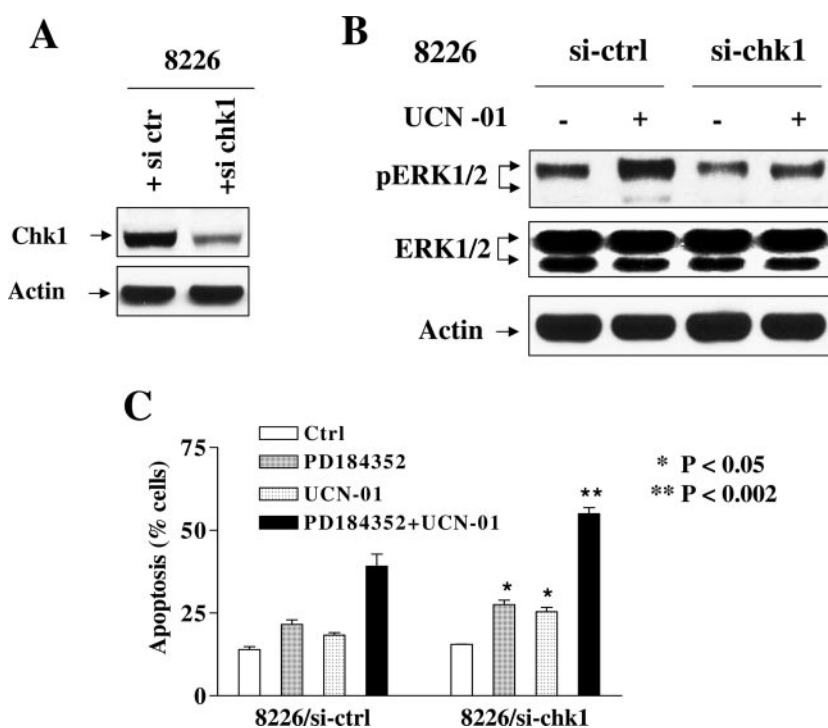


Fig. 5. Knockdown of Chk1 by siRNA does not cause MEK/ERK activation but diminishes UCN-01-induced ERK1/2 phosphorylation. **A**, RPMI 8226 cells were transiently transfected with ds RNAi oligonucleotides directed against Chk1 or nontargeting control siRNA oligonucleotides, respectively, and incubated at 37°C for 24 h, after which cells were lysed and subjected to Western blot analysis to monitor the expression of Chk1. **B**, RPMI 8226 cells transfected with Chk1 siRNA or control nontargeting siRNA were exposed to 150 nM UCN-01 for 24 h, after which cells were lysed and subjected to Western blot analysis to evaluate total and phosphorylated ERK1/2. Each lane was loaded with 20 μ g of protein; blots were stripped and reprobed with antiactin antibodies to ensure equal loading and transfer. Two additional studies yielded equivalent results. **C**, RPMI 8226 cells transfected with Chk1 siRNA or control nontargeting siRNA were treated for 24 h with PD184352 (1 μ M) \pm UCN-01 (100 nM), after which the percentage of apoptotic cells was determined by 7-AAD uptake. Results are representative of three separate experiments. *, significantly greater than the value for cells transfected with control nontargeting siRNA (*, $P < 0.05$; **, $P < 0.002$).

ated lethality in myeloma cells but is not responsible for MEK/ERK1/2 activation by UCN-01. The mechanism by which Cdc2 activation triggers apoptosis has not been clearly defined, but it has been postulated to involve multiple events, including phosphorylation/inactivation of Bcl-2 (Furukawa et al., 2000), phosphorylation/activation of Bad resulting in diminished association with 14-3-3 proteins (Konishi et al., 2002), and induction of premature chromatin condensation and mitotic catastrophe (Niida and Nakanishi, 2006), among others. As noted above, progression of cells through G₂M and mitosis is associated with both activation of Cdc2/cyclin B (Dash and El-Deiry, 2005) and MEK1/2/ERK1/2 (Abbott and Holt, 1999), although the functional relationship between these events has not been clearly established. It is significant

that activation of ERK1/2 by UCN-01 in myeloma cells was observed as early as 3 h after administration of UCN-01, whereas dephosphorylation/activation of Cdc2 was detectable only after 18-h UCN-01 treatment. Thus, from a temporal standpoint, these findings argue against the possibility that ERK1/2 activation results from the Cdc2 activation. This conclusion was further supported by studies using genetic approaches. In particular, ectopic expression of Cdc2, accompanied by an increase in activity, did not increase ERK1/2 phosphorylation/activation; conversely, siRNA-mediated knockdown of Cdc2 expression failed to affect basal levels of phosphorylated ERK1/2 or ERK1/2 activation in response to UCN-01. Such findings are in accord with recent evidence that Cdc2 activation does not increase, and may in

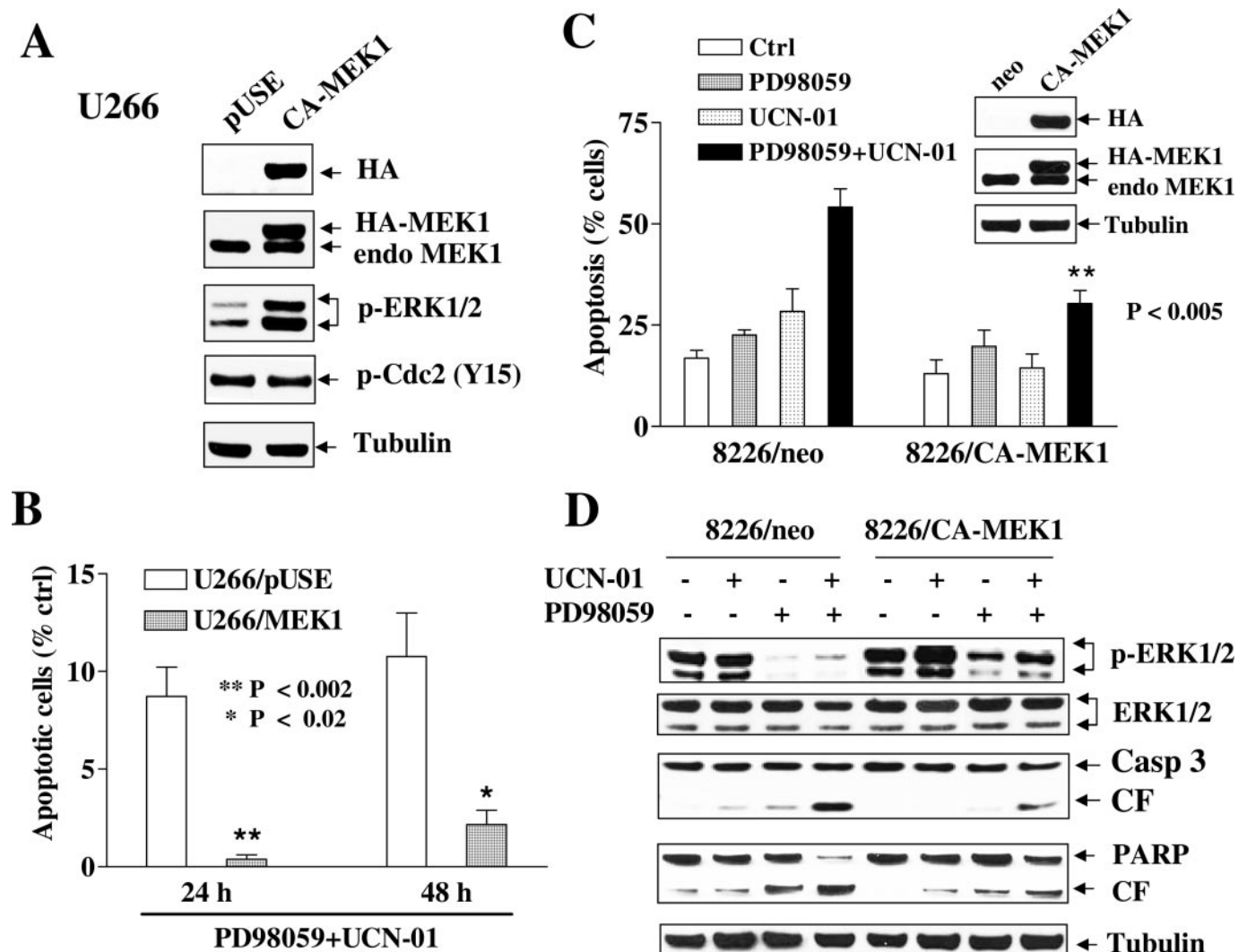


Fig. 6. Ectopic expression of constitutively active MEK1 blocks apoptosis induced by PD98059/UCN-01 without affecting Cdc2 activation. **A** and **B**, U266 cells were transiently transfected with a construct encoding HA-tagged CA MEK1 or its empty vector (pUSEamp) as described under *Materials and Methods*. **A**, after 24-h incubation at 37°C, cells were lysed and subjected to Western blot analysis to monitor expression of MEK1, phospho-ERK and phospho-cdc2. **B**, cells were then treated with 100 nM UCN-01 + 50 μ M PD98059 for 24 h and 48 h, and percentage of death cells was assessed by 7-AAD uptake. Results represent the means \pm S.D. for three separate experiments performed in triplicate. *, significantly lower than the value for cells transfected with empty vector (*, $P < 0.02$; **, $P < 0.002$). **C**, RPMI 8226 cells were stably transfected with a construct encoding HA-tagged CA MEK1 or its empty vector (pUSEamp), and clones expressing HA-tagged MEK1 were selected by Western blot analysis using anti-HA and anti-MEK1 antibodies (inset). Cells were exposed to 100 nM UCN-01 + 50 μ M PD98059 for 24 h, after which apoptosis was assessed by Annexin V-FITC staining and flow cytometry. Results represent the means \pm S.D. for three separate experiments performed in triplicate. *, significantly lower than the value for cells transfected with empty vector ($P < 0.005$). **D**, RPMI 8226 cells stably transfected with CA MEK1 or its empty vector control were treated as described in **C**, after which cells were lysed and subjected to Western blot analysis using the indicated primary antibodies. CF, cleavage fragment. Each lane was loaded with 20 μ g of protein; blots were stripped and reprobed with antitubulin antibody to ensure equal loading and transfer. Two additional studies yielded equivalent results.

fact diminish, ERK1/2 activation induced by epidermal growth factor in HeLa and MDA-MB-468 cells (Dangi and Shapiro, 2005). Thus, although Cdc2 activation is a well-described consequence of UCN-01 exposure (Wang et al., 1995; Dai et al., 2001), the present results, involving both temporal and genetic evidence, argues strongly that UCN-01-mediated MEK1/2/ERK1/2 activation in myeloma cells proceeds through a Cdc2-independent mechanism.

Although activation of MEK1/2/ERK1/2 is unlikely to represent a consequence of UCN-01-mediated Cdc2 activation, it is likely that this event, whatever its cause, attenuates UCN-01 lethality. The mechanism by which MEK1/2/ERK1/2 activation protects cells from apoptosis is also not known with certainty but may involve 1) activation of downstream kinases and transcriptional factors that control the expression of genes, including many antiapoptotic ones; 2) phosphorylation of and interference with the functions of certain proapoptotic proteins such as Bad (Scheid et al., 1999), Bim (Moller et al., 2005), and caspase 9 (Allan et al., 2003); or 3) a combination of these actions. The observation that enforced expression of MEK-CA significantly reduced UCN-01/MEK1 inhibitor lethality clearly demonstrates the protective effect of MEK/ERK activation, in a Cdc2-independent manner, on the drug-induced killing. The protective effect of MEK/ERK activation induced by UCN-01 is substantiated by the recent findings that farnesyltransferase inhibitors, which operate upstream of MEK1/2 by disrupting Ras farnesylation (Pan and Yeung, 2005), also markedly potentiate UCN-01 lethality in human leukemia and myeloma cells in association with MEK1/2/ERK1/2 inactivation (Dai et al., 2005; Pei et al., 2005).

Having ruled out the possibility that Cdc2 activation was responsible for MEK1/2/ERK1/2 activation in UCN-01-treated cells, attention focused on a potential role for perturbations in Chk1 in this phenomenon, because Chk1 lies upstream of Cdc2 signals in response to DNA damage and is one of the UCN-01 targets (Graves et al., 2000; Syljuasen et al., 2005). In this regard, a relationship between checkpoint regulation and ERK1/2 activation has been described previously. For example, it has been shown that in MCF-7 breast cancer cells, enforced expression of BRCA1, an activator of Chk1 (Yarden et al., 2002), is associated with induction of ERK1/2 activation (Yan et al., 2005). It is interesting that inhibition of ERK1/2 under these circumstances by pharmacological or genetic means resulted in a marked increase in apoptosis (Yan et al., 2002). Such findings increase the possibility that dysregulation of Chk1 may be related with cytoprotective MEK1/2/ERK1/2 response to circumvent apoptosis, at least in breast cancer cells. However, the present data argue against the possibility that inactivation of Chk1 by itself is responsible for UCN-01-mediated ERK1/2 activation in cells exposed to this agent. It is interesting that siRNA-mediated knockdown of Chk1, through an as-yet-to-be-determined mechanism, resulted in a modest decline in basal phosphorylated ERK1/2 expression and blunted the capacity of UCN-01 to stimulate ERK1/2 activation, suggesting a possibility of a heretofore unrecognized link between Chk1 and ERK1/2 signaling. Whether this phenomenon reflects the direct involvement of the Chk1 protein in activation of the Raf-1/MEK1/2/ERK1/2 module or reflects a more indirect effect (e.g., mediated by MAP kinase phosphatases) remains to be determined. An alternative possibility is that a gradual

decrease in Chk1 levels resulting from siRNA knockdown may be accompanied by compensatory changes in other pathways that attenuate ERK1/2 activation, the effects of which may therefore differ from those of kinase inhibitors such as UCN-01. In any case, the present results suggest that inhibition of Chk1 by UCN-01, per se, is unlikely to be exclusively responsible for MEK/ERK activation.

Together, the present data suggest that acutely inhibiting Chk1 (i.e., by UCN-01) and down-regulating expression of Chk1 (i.e., by siRNA) may exert divergent effects on the MEK1/2/ERK1/2 cascade. In this context, such findings should be compared with the results of a recent study demonstrating that Chk1 siRNA mimicked the actions of Chk1 inhibitors such as UCN-01 and CEP-3891 in phosphorylating diverse ATR targets, including H2AX, smc1, RPA, and Chk1 (Syljuasen et al., 2005). These observations are consistent with the notion that Chk1 activation may be required to prevent aberrant DNA replication initiation and damage. However, in the latter study, the impact of silencing Chk1 (by siRNA) on the response of cells to UCN-01 was not examined. The disparate effects of siRNA-mediated Chk1 knockdown and UCN-01 exposure on MEK1/2/ERK1/2 activation observed here suggest that UCN-01 actions other than or in addition to inactivation of Chk1 are responsible for this phenomenon. It is noteworthy that Chk1 knockdown by siRNA sensitized myeloma cells to UCN-01 lethality, particularly when the latter was combined with MEK inhibitors. One possible explanation for this finding is that Chk1 knockdown by siRNA was incomplete, raising the possibility that residual Chk1 activity might be more susceptible to inhibition by UCN-01.

In summary, the present findings indicate that although the cell cycle dysregulatory actions of UCN-01, particularly induction of Cdc2, are unlikely to be responsible for activation of the MEK1/2/ERK1/2 cascade triggered by this agent in myeloma cells, these events nevertheless contribute functionally to the lethality of the UCN-01/MEK1/2 inhibitor regimen. In addition, diminished Chk1 expression did not by itself induce ERK1/2 activation; instead, it attenuated UCN-01-mediated stimulation of ERK1/2 activity and increased the susceptibility of cells to these agents. Thus, the present observations provide evidence that whatever the primary cause(s) of MEK1/2/ERK1/2 activation induced by UCN-01, this response plays a significant cytoprotective role in limiting UCN-01-mediated lethality associated with Cdc2 activation. A key question remaining to be resolved is the identification of the precise mechanism by which UCN-01 activates MEK1/2/ERK1/2 in the presence of Chk1. Whether this reflects cooperation/cross-talk between Chk1 inhibition and modulation of pyruvate dehydrogenase kinase 1/Akt, PKC isoforms, CDKs, or other as-yet-to-be-identified targets of UCN-01 remains to be determined. Therefore, studies designed to address these issues are currently in progress.

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