

Defective p53 signaling in p53 wild-type tumors attenuates p21^{waf1} induction and cyclin B repression rendering them sensitive to Chk1 inhibitors that abrogate DNA damage-induced S and G₂ arrest

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Abstract

DNA damage induces cell cycle arrest to provide time for repair and enhance cell survival. The Chk1 inhibitor 7-hydroxystaurosporine (UCN-01) can overcome both S and G₂ arrest and drive cells through a lethal mitosis. S-phase arrest induced by the topoisomerase I inhibitor SN38 results from activation of Chk1 and degradation of Cdc25A phosphatase that occurs independent of p53 status. However, p53-mediated induction of p21^{waf1} and repression of cyclin B prevent abrogation of S and G₂ arrest, respectively. Surprisingly, incubation of MCF10A immortalized breast cells with UCN-01 fails to elevate Cdc25A protein due to p53-mediated inhibition of Cdc25A transcription. Suppression of p21^{waf1} in MCF10A cells overcame this transcriptional inhibition, and the S-phase-arrested cells became sensitive to UCN-01, although they now arrested in G₂ as cyclin B expression remained suppressed. We also compared the response of p53 wild-type tumors to the combination of SN38 and UCN-01. In CAKI-1, U87MG, and SUM102, SN38 induced p21^{waf1} and the cells were resistant to UCN-01. In contrast, HCT116 and MCF7 cells had markedly attenuated induction of p21^{waf1} and failed to repress cyclin B. Accordingly, these cells were susceptible to UCN-01-mediated abrogation of both S and G₂ arrest. SN38

induced expression of another p53-inducible gene, 14-3-3σ, suggesting selective dysregulation of p53 response genes. In summary, several cell lines commonly considered wild-type for p53 appear to have defects in expression of selected p53 response genes following DNA damage, and this makes them sensitive to the combination of DNA damage plus Chk1 inhibitor. [Mol Cancer Ther 2008;7(2):252–62]

Introduction

Cell cycle checkpoints are critical for the protection of cells from DNA damage. The checkpoint-mediated arrest permits time for DNA repair, after which a cell can recover. In the case of cancer, activation of the checkpoints protects the tumor cells from many anticancer drugs. This has led to the search for novel agents that can inhibit the checkpoint, thereby enhancing death by driving cells through the cell cycle before they have adequately repaired the damage. This strategy was initially suggested in experiments with caffeine, which forces G₂-arrested cells through a lethal mitosis (1). Caffeine also inhibits S-phase arrest, inducing cells to progress to G₂ before lethal mitosis (2). The target for caffeine is recognized as ATM and ATR (3). Intriguingly, abrogation of damage-induced S and G₂ arrest occurs preferentially in p53-defective cells (4, 5). Hence, p53 can protect cells either by directly inducing a G₁ arrest or by reinforcing S and G₂ arrest. This approach may selectively kill p53-defective tumors without increasing toxicity to the patient.

Unfortunately, patients cannot tolerate caffeine at concentrations required to abrogate cell cycle arrest. We discovered 7-hydroxystaurosporine (UCN-01) as a novel checkpoint inhibitor that is much more potent than caffeine and abrogates both S and G₂ arrest preferentially when p53 is defective (6, 7). The critical target for UCN-01 is Chk1 as the low concentrations used do not inhibit other reported checkpoint proteins Chk2 and MAPKAPK2 (8–11).¹ The concentrations required to inhibit Chk1 can be exceeded in animal models, although its use as a therapeutic agent in patients may be compromised by avid binding to human plasma proteins (12). Many pharmaceutical companies are currently testing alternate compounds as checkpoint inhibitors (13).

We recently extended our investigations on checkpoint regulation by assessing the effect of p53 in isogenic cell lines (14). Incubation of immortalized mammary epithelial

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¹ A. Poh and A. Eastman, unpublished observations.

MCF10A cells (p53 wild-type) with the topoisomerase I inhibitor SN38 induces arrest at S or G₂ depending on drug concentration. SN38 was selected for these studies because its primary damage occurs when a replication complex collides with an inhibited topoisomerase I resulting in a DNA double-strand break, and the S-phase dependence of this event precludes a confounding p53-mediated G₁ arrest. Addition of UCN-01 did not overcome this S-phase arrest in MCF10A cells, although it abrogated arrest in various p53-defective cells. MCF10A cells suppressed for p53 still exhibited SN38-induced S arrest, but on addition of UCN-01 they progressed through S and through a lethal mitosis. Importantly for the current work, through expression of a dominant-negative fragment of p53, we also derived a MCF10A cell line that was defective in p53-mediated transactivation of p21^{waf1}, but it retained p53-mediated repression of cyclin B. This cell line was sensitive to UCN-01-induced S-phase abrogation but remained arrested in G₂. We therefore proposed that p21^{waf1} protected S-phase cells from UCN-01, but in addition, repression of cyclin B protected G₂ cells from undergoing a lethal mitosis.

Several other investigators have reported that UCN-01 can abrogate arrest independent of p53 status and this questions the importance of p53 in protecting cells from checkpoint abrogation (15, 16). However, there is a critical difference between these studies in that UCN-01 was added either before or within 3 h of initiating damage. In this schedule, the p53 protective response is not activated before inhibiting Chk1. We have confirmed that concurrent incubation with UCN-01 prevents arrest of MCF10A cells¹ as has been shown with p53 wild-type HCT116 cells (10). Additional evidence from HCT116 cells shows that, even after arrest has been induced, UCN-01 can abrogate arrest despite activation of p53 (10). This was explained by the observation that UCN-01 can repress expression of p21^{waf1}, thereby overcoming the protection it provides. In contrast, it has been shown that damaged HCT116 cells will eventually enter an aberrant mitosis in the absence of a checkpoint inhibitor and despite the presence of p21^{waf1} (17).

These latter reports all address the role of p53 in tumor cells, whereas our recent investigation of the role of p53 focused on the nontumorigenic MCF10A cell line because of our desire to understand whether a patient's normal cells might be protected from checkpoint inhibitors. However, if some p53 wild-type tumors are sensitive to UCN-01, then this would extend the efficacy of checkpoint inhibitors to an even greater number of tumors. With this possibility in mind, we have revisited the effect of UCN-01 in p53 wild-type tumors and find that some are sensitive to Chk1 inhibition. Importantly, we find that the sensitivity to S-phase abrogation correlates with a defective induction of p21^{waf1}. This appears selective for p21^{waf1} as another p53 response gene was rapidly up-regulated in these cells. The failure of p53 wild-type tumors to remain arrested in G₂ correlates with their inability to repress cyclin B. Accordingly, it appears that

some tumors with wild-type p53 have defects in p53 signaling that can be observed as dysregulation of both its transactivation and repression functions.

Materials and Methods

Chemicals

SN38, the active metabolite of the topoisomerase I inhibitor irinotecan, was provided by Pfizer Global. UCN-01 was provided by the National Cancer Institute. Both drugs were dissolved in DMSO at >1,000 times the final concentration used in experiments.

Cell Culture

Cell lines were obtained from the American Type Culture Collection: MCF10A immortalized breast cancer line, MCF7 breast cancer, U87MG glioblastoma, CAKI-1 renal cell carcinoma, and MDA-MB-231 p53 mutant breast cancer. The SUM102 breast cancer cell line was obtained from Asterand. The HCT116 colorectal carcinoma cell lines were obtained from Dr. Kunkel (National Institute of Environmental Health Sciences). Where noted, we used HCT116 and HCT116/p21^{-/-} cells provided by Dr. Vogelstein (Johns Hopkins University).

Most of the cell lines were grown in DMEM/F-12 supplemented with 10% fetal bovine serum plus antibiotic/antimycotic. For MCF10A cells, the medium was supplemented with 8 µg/mL insulin, 20 ng/mL epidermal growth factor, and 500 ng/mL hydrocortisone. SUM102 were maintained in defined serum-free medium according to the supplier's recommendation. HCT116 cells were maintained in McCoy's 5a medium supplemented with serum and antibiotics as above.

Suppression of p21^{waf1} was achieved using a short hairpin RNA sequence cloned into pSilencer and provided by Dr. Shigeki Miyamoto (University of Wisconsin; ref. 18). The plasmid was cotransfected into MCF10A cells together with pcDNA3.1 to facilitate selection of transformants in G418; the resulting cell line was termed MCF10A/Δp21. Multiple colonies were screened to select several with maximum suppression of p21^{waf1}, and similar results have been obtained with at least two clones.

Cell Cycle Analysis

Cell cycle analysis was done as described previously (2, 14). Cyclin B was assessed by incubating ethanol-fixed cells with FITC-conjugated anti-cyclin B antibody (PharMingen) before addition of RNase and propidium iodide (19). Expression of p21^{waf1} and geminin required fixing cells in 2% formalin for 15 min followed by storage in 70% ethanol. Fixed cells were stained with either FITC-conjugated anti-p21^{waf1} antibody (Calbiochem) or anti-geminin antibody (FL209; Santa Cruz Biotechnology) followed by an Alexa 488-conjugated anti-rabbit antibody (Invitrogen). Cells were then incubated with RNase and propidium iodide.

Immunoblotting

Cells were rinsed with PBS and lysed by addition of Laemmli sample buffer. Samples were immediately boiled for 5 min and stored at -20°C. Proteins were separated by

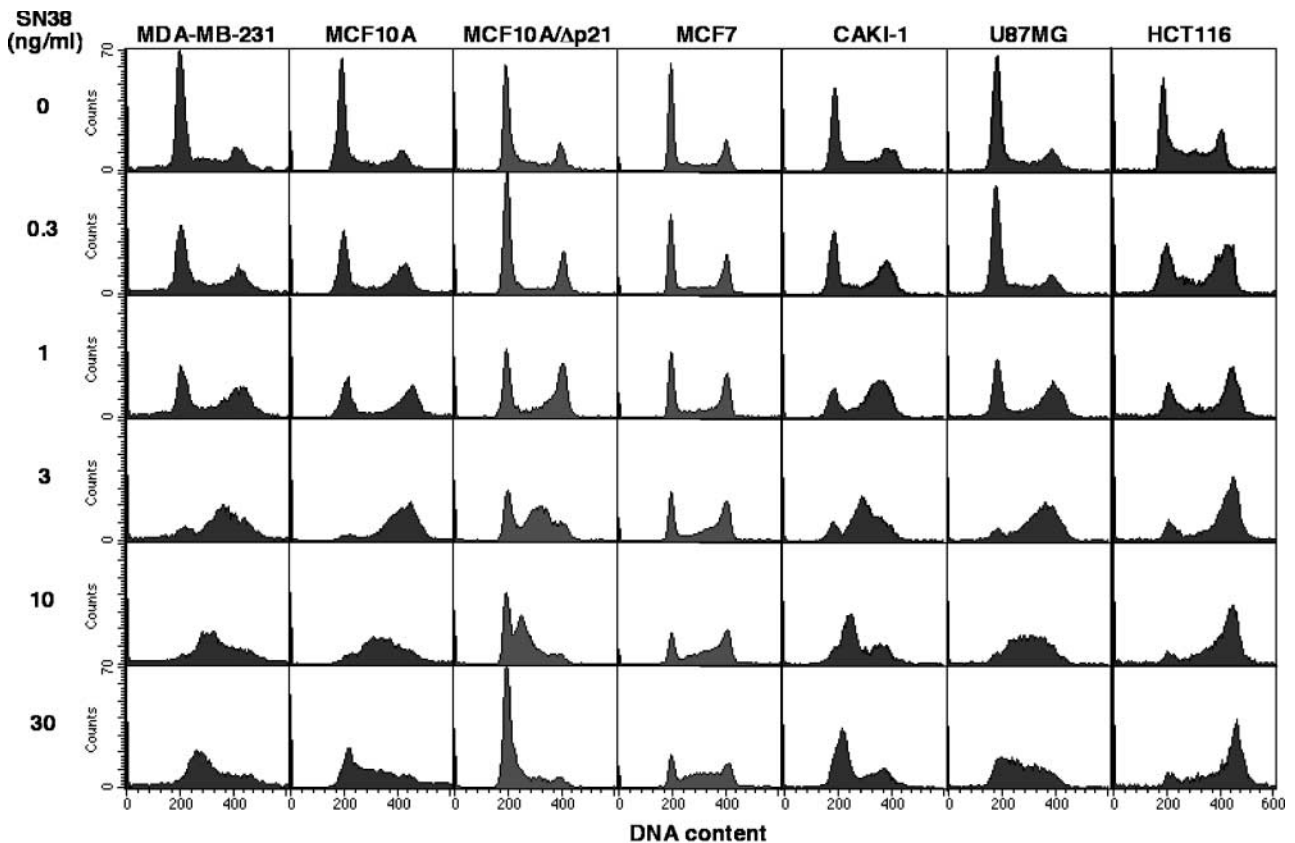


Figure 1. Effect of SN38 on cell cycle arrest in the p53-defective MDA-MB-231 cells and a panel of p53 wild-type cell lines. Cells were incubated with 0 to 30 ng/mL SN38 for 24 h, harvested, and assayed for DNA content by flow cytometry.

SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk in TBS, 0.1% Tween 20, and probed with antibody overnight at 4°C [cyclin B1 (GNS1), p53 (DO-1), p21^{waf1} (C19), Cdc25A (F6; Santa Cruz Biotechnology); phosphoserine 15-p53(16G8), phosphoserine 20-p53, Cell Signaling; β-actin (Ab-1; Oncogene), and 14-3-3σ (Immuno-Biological Laboratories)]. Subsequently, membranes were washed in TBS, 0.1% Tween 20, and incubated with secondary antibody conjugated to horseradish peroxidase (Bio-Rad). Proteins were visualized by enhanced chemiluminescence (Amersham).

PCR

The transcript level of Cdc25A was assessed by semi-quantitative PCR. Total RNA was isolated using Qiagen's RNeasy Mini kit. Synthesis of cDNA was done with 1.5 μg RNA. The primers (20) gave a single band of 272 bp when amplified for 35 cycles with 30 s each at 94°C, 60°C, and 72°C. Initial experiments determined the concentration of cDNA that permitted semiquantitative comparisons, and the experiments shown were all done within this range.

Results

DNA Damage-Induced Cell Cycle Arrest

Our previous studies using isogenic immortalized MCF10A cells have shown that p53 can prevent UCN-01

from abrogating S-phase arrest induced by SN38 (14). The goal of this study was to extend these observations to p53 wild-type tumors to establish whether p53 could also prevent abrogation of arrest in cells with a transformed phenotype. We first compared the ability of SN38 to induce arrest in a panel of p53 wild-type cell lines; for comparison, we also show the arrest induced in the p53 mutant MDA-MB-231 cell line (Fig. 1). Most of the cell lines accumulated in G₂ 24 h after addition of 1 ng/mL SN38 or at late, middle, or early S phase at higher concentrations. The notable exception was HCT116 cells that failed to arrest in S phase but accumulated in G₂ at all concentrations of SN38. These cells have a defect in another checkpoint pathway (Mre11:Rad50:Nbs1), which may explain their inability to arrest in S phase (21).

Abrogation of Cell Cycle Arrest by UCN-01

We next asked whether the Chk1 inhibitor UCN-01 would abrogate SN38-induced arrest in the various p53 wild-type cell lines. Cells were incubated with 10 ng/mL SN38 for 24 h to induce primarily a S-phase arrest (except for HCT116, which arrest in G₂). On removal of SN38, the MDA-MB-231 cells progress slowly to G₂ over the following 24 h (Fig. 2). The addition of 15 nmol/L UCN-01 causes abrogation of S-phase arrest within 6 h (30-h time point), whereas the majority of cells abrogated G₂ arrest within 24 h (48-h time point). The resulting cells show

fragmented nuclei when observed under the microscope indicative of mitotic catastrophe and a heterogeneous DNA content when assayed by flow cytometry.

Whereas 15 nmol/L UCN-01 abrogates arrest in MDA-MB-231 cells, concentrations as high as 500 nmol/L fail to abrogate arrest in MCF10A cells (19). The experiments shown in Fig. 2 were done with 15 to 30 nmol/L UCN-01, whereas concentrations up to at least 100 nmol/L were done in all cell lines with similar results. The CAKI-1 cell

line responded to UCN-01 in a manner similar to that for MCF10A cells, that is, the majority of cells remained arrested in S phase for at least 24 h after removal of SN38, with little effect from addition of UCN-01. Another p53 wild-type breast cancer cell line, SUM102, responded in an identical manner (data not shown). The U87MG cell line was similar, although there was a slight shift of the cells to G₂ 24 h after addition of UCN-01. In contrast, the MCF7 cells progressed to G₂ within 24 h of removing SN38; the

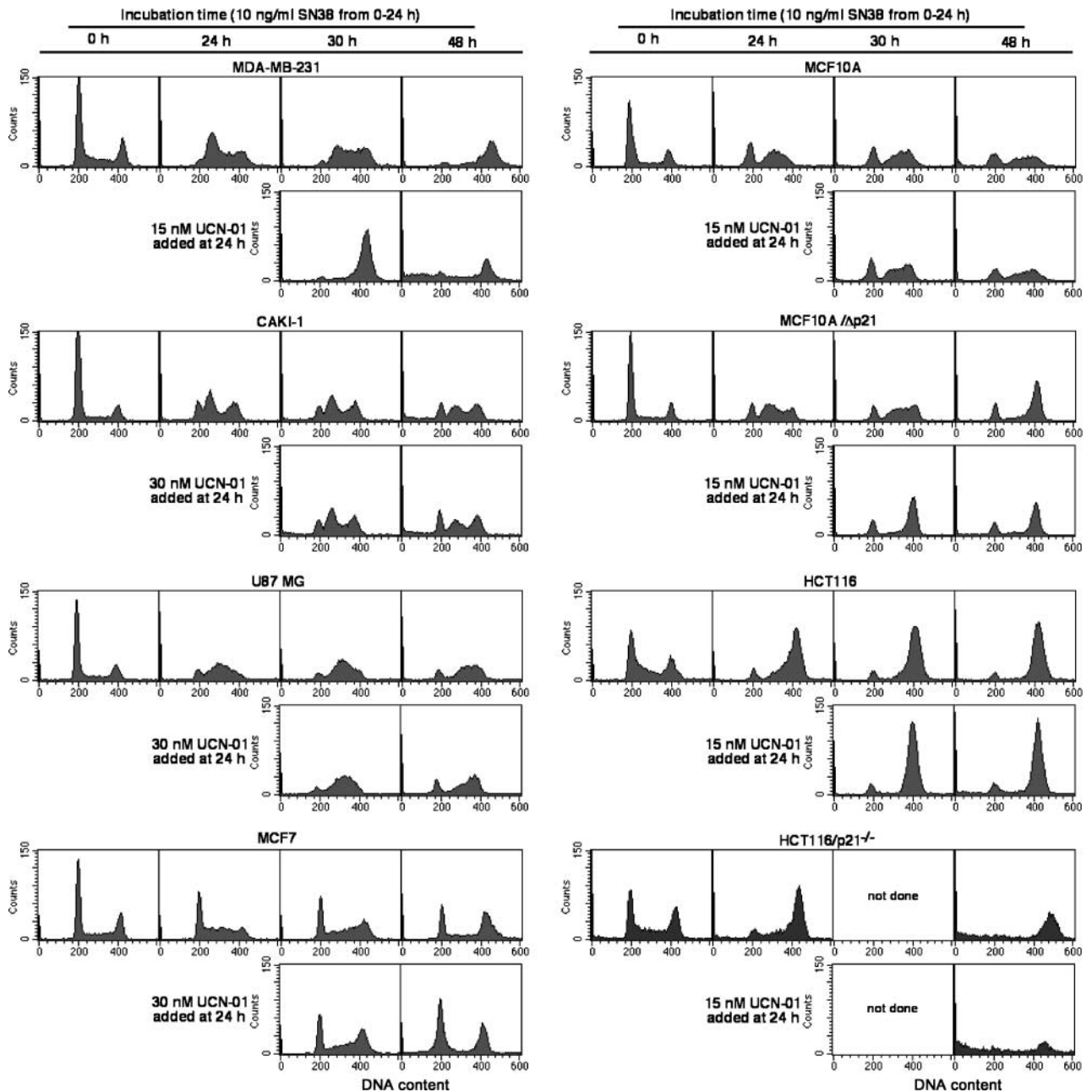


Figure 2. Comparison of the efficacy of UCN-01 to abrogate SN38-induced S and G₂ arrest. Each cell line was incubated with 10 ng/mL SN38 for 24 h; medium was removed, and fresh medium with or without UCN-01 was added for an additional 6 or 24 h. Cells were harvested at the indicated times and DNA content was assessed by flow cytometry. UCN-01 alone at the concentrations used here had no effect on cell cycle distribution (ref. 19; data not shown).

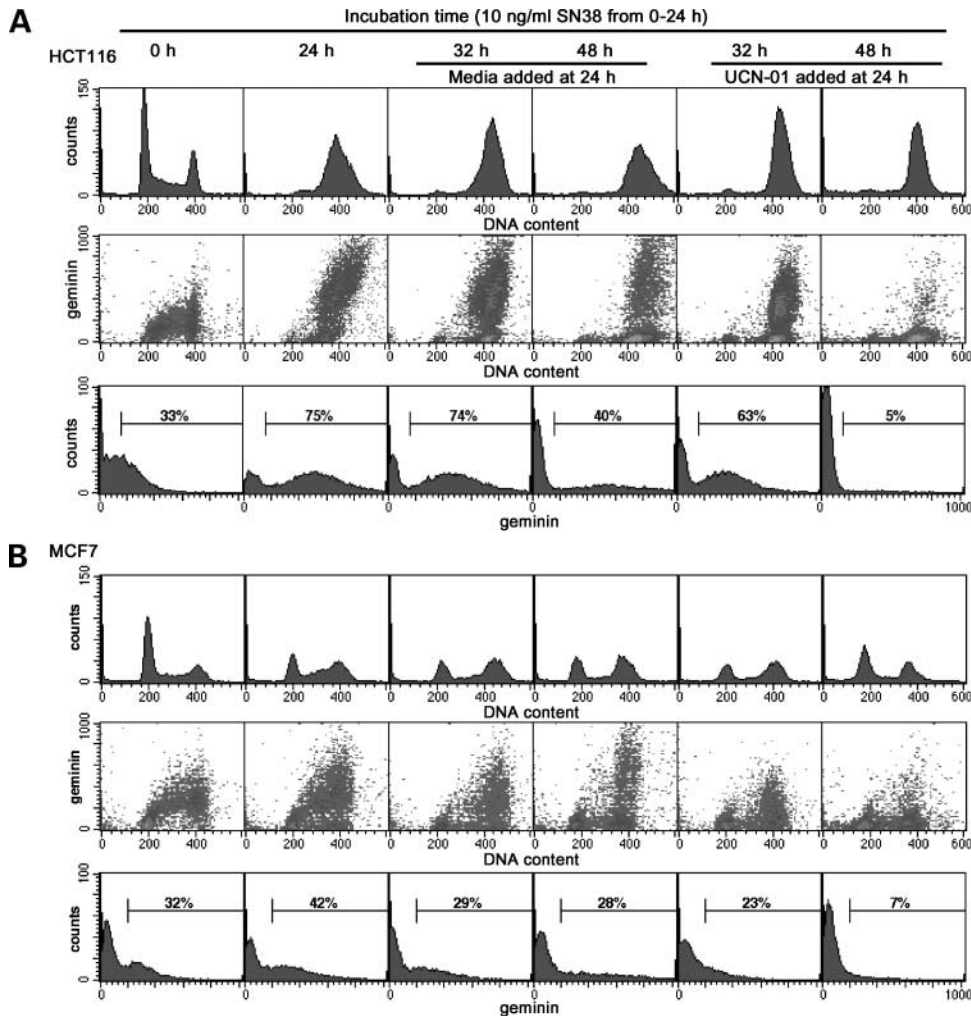


Figure 3. Discrimination of cells in G₂ versus M phase by their expression of geminin. HCT116 (A) and MCF7 (B) cells were incubated with 10 ng/mL SN38 for 24 h. The drug was removed and cells incubated for an additional 6 or 24 h in either fresh medium or 15 nmol/L UCN-01. Cells were harvested at the indicated times and stained for geminin and DNA. The histogram statistics (*bottom*) reflect the percentage of cells expressing geminin, the remainder of the cells being in either G₁ or M.

addition of UCN-01 accelerated progression through S and enhanced the proportion of cells that underwent mitosis. Finally, the HCT116 cells, which arrested primarily in G₂ phase on SN38, showed no apparent progression through mitosis on addition of UCN-01. However, microscopic analysis showed a large proportion of cells with extensive nuclear fragmentation reminiscent of mitotic catastrophe, and this occurred in the majority of cells following incubation with UCN-01 (Supplementary Fig. S1).² Microscopic analysis of MCF7 cells also showed a significant but lower proportion of the cells with fragmented nuclei (data not shown).

We investigated this apparent mitotic catastrophe further using an antibody to geminin that detects cells in S and G₂. Geminin is degraded by the anaphase-promoting complex in mitosis and can therefore resolve the G₂-M population observed by flow cytometry (22). By two-dimensional flow

cytometry, asynchronous cells showed few cells in G₂-M with low geminin, consistent with the majority of these cells being in G₂, whereas mitotic cells normally undergo cytokinesis rapidly (Fig. 3). DNA damage caused an increase in the number of geminin-positive cells, and most of the cells in G₂-M were clearly positive, confirming they were in G₂. Following removal of SN38, there was a marked increase in geminin-negative cells with G₂-M DNA content and this was dramatically enhanced by incubation with UCN-01. In the case of the HCT116 cells, the majority of cells have undergone mitosis, yet they remain with a tetraploid DNA content. Many of the MCF7 cells appeared to have undergone cytokinesis, but those remaining with a tetraploid DNA content also show degradation of geminin. Hence, cells can undergo mitosis but fail to undergo cytokinesis, and in the case of HCT116 cells, this can involve the entire population.

Effect of p21^{waf1} on Cell Cycle Arrest

We suggested previously that p21^{waf1} could protect cells from UCN-01-induced S-phase abrogation (14). As all the cells being studied here contain wild-type p53, it is expected that DNA damage should increase p21^{waf1}.

² Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

Following a 24-h incubation with SN38, p21^{waf1} was significantly induced in S-phase-arrested MCF10A cells, and this remained elevated on removal of SN38 even in the presence of UCN-01 (Fig. 4). Very similar results were obtained with CAKI-1 and U87MG cells (data not shown). These results are consistent with these three cell lines remaining arrested primarily in S phase throughout the timeframe of the experiment. In contrast, SN38 induced a very low level of p21^{waf1} in MCF7 and HCT116 cells. On removal of SN38 at 24 h, both of these cell lines showed a progressive increase in p21^{waf1}, which by 48 h was equivalent to or greater than that observed in the MCF10A cells. However, despite the presence of p21^{waf1} by 48 h, both MCF7 and HCT116 cell lines were still able to undergo mitosis as shown above.

The extent of p21^{waf1} induction was also compared by two-dimensional flow cytometry (Supplementary Fig. S2).² S-phase-arrested MCF10A cells show a significant increase in p21^{waf1} that is maintained following removal of SN38, consistent with the results seen by Western blotting (Fig. 4). Both MCF7 and HCT116 cells show almost no detectable p21^{waf1} after 24-h incubation with SN38, but this increased dramatically with time in both the presence and the absence of UCN-01. These results confirm the delayed kinetics of induction of p21^{waf1} in the HCT116 and MCF7 cells. Furthermore, the low level of p21^{waf1} in S-phase-arrested MCF7 cells may explain why these cells are able to progress to G₂, whereas high p21^{waf1} may prevent progression of MCF10A cells. The results further emphasize that p21^{waf1} remains elevated 24 h after addition of

UCN-01, a time point at which both MCF7 and HCT116 cells have undergone mitosis.

To further investigate the failure of HCT116 cells to undergo cytokinesis, we did parallel experiments in HCT116 cells that have been deleted for p21^{waf1}. These cells also arrested primarily in G₂ after incubation with SN38 and underwent a mitotic catastrophe on further incubation in UCN-01 (Fig. 2). However, these cells showed extensive fragmentation following mitosis comparable with that observed in the p53 mutant MDA-MB-231 cells. Hence, it appears that although the high level of p21^{waf1} is unable to prevent mitosis, it can prevent cytokinesis.

p21^{waf1} Is Required for Maintenance of S-Phase Arrest

We next used short hairpin RNA to suppress p21^{waf1} in MCF10A cells and determine whether it was the critical p53 response gene that prevented UCN-01-induced S-phase progression. The resulting MCF10A/Δp21 cells still arrested in S phase when damaged (Fig. 1). UCN-01 forced them to progress to G₂ where they remained with no evidence of mitotic catastrophe (Fig. 2). The effectiveness of repression of p21^{waf1} after the 24-h incubation with SN38 is shown in Fig. 4. However, by the end of the experiment (48 h), the short hairpin RNA appeared to have lost its ability to completely repress p21^{waf1}, perhaps because extensive DNA damage impedes the short hairpin RNA expression or function.

The mechanism by which p21^{waf1} prevents S-phase progression is through inhibition of a cyclin/Cdk complex, such as cyclin E/Cdk2. However, we have noted previously

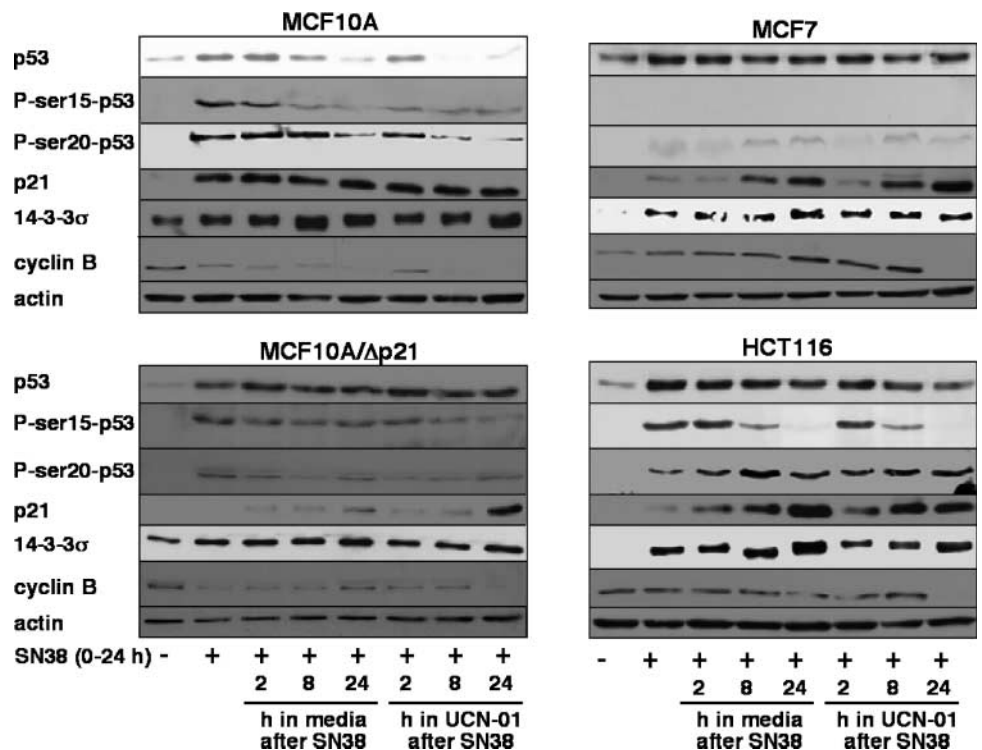


Figure 4. Analysis of the p53 response following incubation of cells with SN38. Each of the four indicated cell lines were incubated with 10 ng/mL SN38 for 24 h. The drug was removed and cells incubated for an additional 2, 8, or 24 h in either fresh medium or 15 nmol/L UCN-01. Cells were harvested and analyzed by Western blotting for expression of the indicated proteins.

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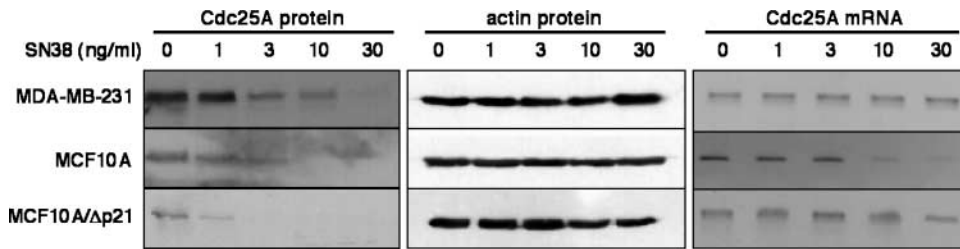


Figure 5. DNA damage represses transcription of Cdc25A mRNA in a p21^{waf1}-dependent manner. The three cell lines were incubated with 0 to 30 ng/mL SN38 for 24 h. Cell lysates were analyzed for expression of Cdc25A protein. RNA was simultaneously prepared from parallel cultures and semi-quantitative PCR was used to assess the relative levels of CDC25A mRNA.

that UCN-01-mediated inhibition of Chk1 in MCF10A cells fails to induce accumulation of Cdc25A protein, a necessary step required for activation of cyclin E/Cdk2 (14). We therefore investigated the effect of p21^{waf1} on transcription of Cdc25A. Incubation with SN38 reduced Cdc25A protein but had no effect on the Cdc25A mRNA level in MDA-MB-231 cells (Fig. 5). In MCF10A cells, SN38 reduced both protein and mRNA levels, showing that DNA damage can inhibit Cdc25A at the transcriptional level in p53 wild-type cells. In the MCF10A/Δp21 cells, SN38 reduced Cdc25A protein but failed to suppress Cdc25A mRNA, consistent with their ability to progress through S phase when incubated with UCN-01. Accordingly, it appears the primary mechanism by which p21^{waf1} prevents S-phase progression is through transcriptional repression of Cdc25A.

Repression of Cyclin B Maintains G₂-Phase Arrest

The resistance of the MCF10A/Δp21 cells to UCN-01-mediated abrogation of G₂ arrest is reminiscent of our previous work in MCF10A/EB cells, which also remained arrested in G₂; this cell line has lost p53-mediated transactivation of p21^{waf1} but retained p53-mediated repression of cyclin B (14). Accordingly, we investigated the levels of cyclin B as a consequence of DNA damage. Two approaches were used: Western blotting and two-dimensional flow cytometry. The Western analysis shows repression of cyclin B in damaged MCF10A and MCF10A/Δp21 cells (Fig. 4). Cyclin B was also repressed in CAKI-1 and U87MG cells (data not shown). In contrast, MCF7 and HCT116 cells failed to repress cyclin B, except 24 h after UCN-01, at which time the cells have undergone mitosis where cyclin B is degraded by the anaphase-promoting complex (concurrent with the degradation of geminin seen in Fig. 4). Analysis by flow cytometry provides a much more detailed analysis of the expression of cyclin B as it shows the proportion of cells in each cell cycle phase with elevated or repressed protein (Fig. 6). Although cyclin B normally begins to accumulate in S phase, the damaged MCF10A cells show dramatic repression with most of the cells arrested in S phase exhibiting undetectable levels of cyclin B. It is interesting that the few cells remaining in S phase with elevated cyclin B progress to G₂ following removal of SN38, particularly when incubated with UCN-01, suggesting these cells have not fully activated the p53 response at this time. By 48 h, even these cells have repressed cyclin B. In contrast, both MCF7 and HCT116 cells show accumulation of cyclin B in the majority of damaged cells, and this only decreases once the cells have undergone mitosis 24 h after addition of UCN-01.

The MCF10A/Δp21 cells show a profile of cyclin B expression that is comparable with that seen in the parental MCF10A cells, that is, the majority of cells in S phase have low levels of cyclin B (Fig. 6). On removal of SN38, UCN-01 induced rapid progression to G₂, thereby reiterating that p21^{waf1} is sufficient to prevent this progression. Although UCN-01 appears to induce a transient increase in cyclin B, by the time the cells are arrested in G₂, they have almost completely repressed cyclin B, consistent with their failure to undergo mitosis.

Differential Regulation of p53 Response Genes

Considering the dramatic difference between the cell lines with respect to the kinetics of induction of p21^{waf1}, we investigated whether the attenuated induction in MCF7 and HCT116 cells correlated with an attenuated activation of p53. Following incubation with SN38, the MCF10A cells showed strong phosphorylation of p53 at both serines 15 and 20 (Fig. 5). This phosphorylation decreased with time thereafter along with a concomitant decrease in p53 probably due to p53-mediated induction of HDM2 that stimulates p53 proteolysis. SN38 induced a similar response in HCT116 cells. Hence, the kinetics of phosphorylation of p53 in HCT116 cells does not correlate with the attenuated induction of p21^{waf1}. Surprisingly, the MCF7 cells showed no detectable phosphorylation on serine 15, and only weak phosphorylation on serine 20, yet they still elicited an apparent p53 response (see below).

Considering the lack of correlation between p53 activation and induction of p21^{waf1}, we assayed expression of another p53 response gene, 14-3-3σ. There was a significant difference in basal levels of 14-3-3σ in the various cell lines, with MCF10A showing a very high level compared with HCT116 and MCF7 (Fig. 5). The high basal level in MCF10A correlates with their expression of the p53 homologue p63 (23), which may be an alternate means to up-regulate 14-3-3σ. On incubation with SN38, 14-3-3σ was strongly induced in MCF7 and HCT116 cells consistent with the activation of p53. Importantly, this induction occurred during incubation with SN38 with no significant increase following removal of drug. Hence, the fast kinetics of induction of 14-3-3σ in HCT116 and MCF7 cells is very different than the attenuated kinetics observed for p21^{waf1}, suggesting differences in the mechanism by which these two genes are regulated. These results suggest that the p53-mediated regulation of p21^{waf1} and cyclin B is aberrant in MCF7 and HCT116 cells, which makes them sensitive to inhibitors of Chk1.

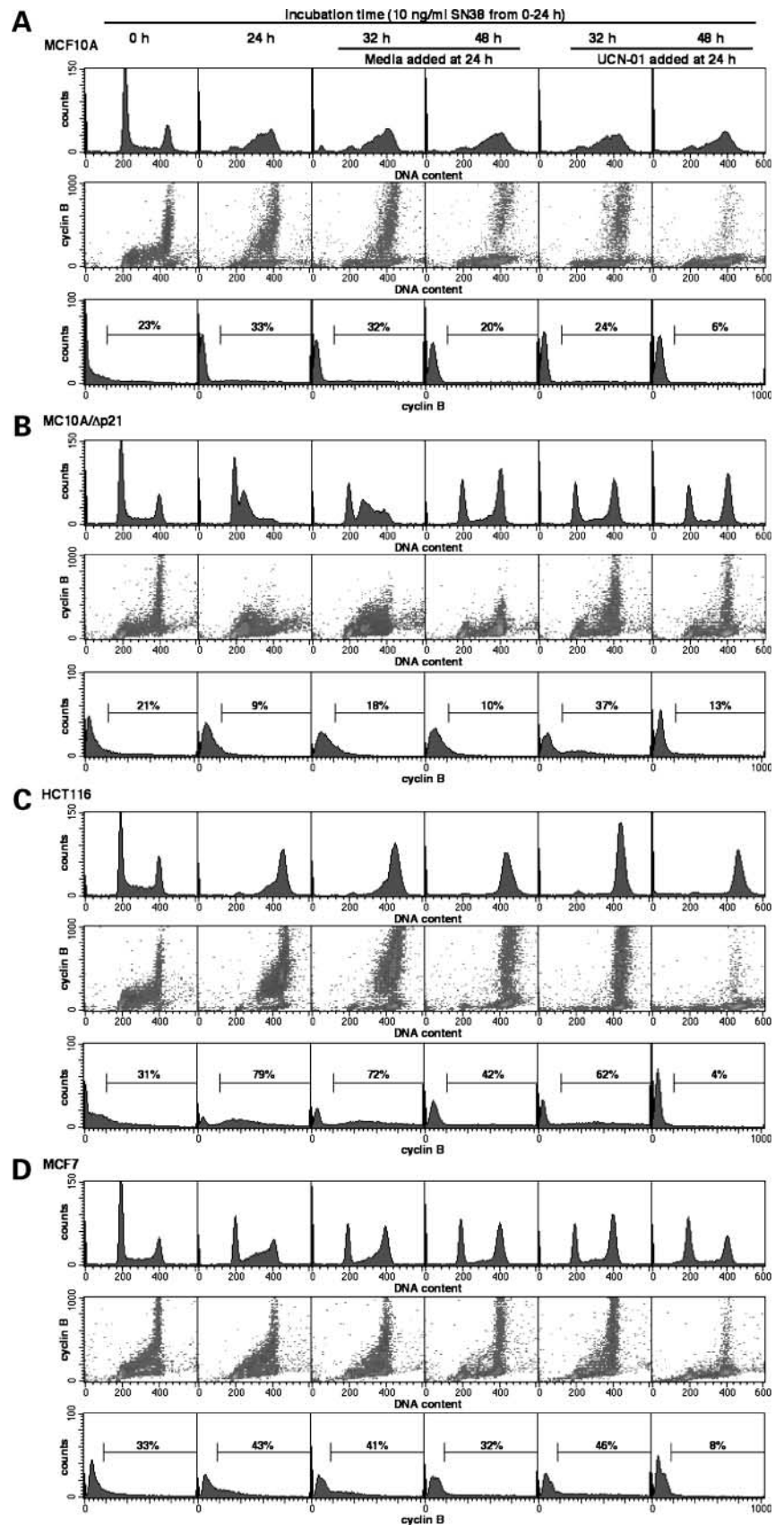


Figure 6. Analysis of cyclin B expression by flow cytometry. MCF10A (A), MCF10A/Δp21 (B), HCT116 (C), and MCF7 (D) cells were incubated with 10 ng/mL SN38 for 24 h. The drug was removed and cells incubated for an additional 6 or 24 h in either fresh medium or 15 nmol/L UCN-01. Cells were harvested at the indicated times and stained for cyclin B and DNA. The histogram statistics (*bottom*) reflect the percentage of cells expressing cyclin B.

Discussion

Loss of the p53 tumor suppressor is frequently considered critical for the survival of tumor cells, and reintroduction or activation of p53 has been proposed as an approach to induce apoptosis. However, p53 can induce both apoptosis and cell cycle arrest. The fate of a cell in response to DNA damage is context specific with most tissues showing a preference for cell cycle arrest, whereas thymocytes and splenocytes are more susceptible to apoptosis (24). It has therefore been suggested that defects in p53 may be an Achilles heel for the tumor, and the development of checkpoint inhibitors attempts to take advantage of this defect to selectively kill tumor cells compared with normal cells (25). DNA damage activates Chk1 and arrests cells in S and G₂ phase independent of p53 status. Our previous studies showed that the Chk1 inhibitor UCN-01 abrogates S and G₂ arrest in p53 mutant MDA-MB-231 cells but not in the p53 wild-type MCF10A cells (19). The role of p53 was subsequently confirmed in isogenic MCF10A cells (14). The current series of experiments was initiated because several authors had failed to observe a role for p53 in protecting cells from UCN-01 (15, 16). We established the importance of sequential rather than concurrent drug administration because the latter schedule inhibits Chk1 before activation of the protective p53 response. During those studies, we also realized that some p53 wild-type cell lines are sensitive to UCN-01, and the experiments reported here focus on explaining this observation.

Several p53 wild-type tumors investigated here, CAKI-1, U87MG, and SUM102, responded to the combination of SN38 and UCN-01 in the same way as the immortalized MCF10A cells showing that resistance to UCN-01 is not unique to nontumorigenic cell lines. Specifically, these cells all arrested in S and/or G₂ depending on the concentration of SN38, and UCN-01 failed to abrogate arrest. These cells up-regulated p21^{waf1} and suppressed cyclin B in response to SN38, two expected consequences of activation of a p53 response. In marked contrast, the HCT116 and MCF7 cells remained sensitive to UCN-01, exhibited very little induction of p21^{waf1} immediately following SN38, and failed to repress cyclin B. At longer times after removal of SN38, both of these cell lines did elevate p21^{waf1}, but this was inadequate to prevent the onset of mitosis. This observation is consistent with a previous report that expression of p21^{waf1} in HCT116 cells is insufficient to prevent mitosis although it did arrest cells in the subsequent tetraploid G₁ state (17). The ability to undergo mitosis also reflected the failure of these cells to repress cyclin B. Hence, the sensitivity of these cells to UCN-01 is explained by an attenuated induction of p21^{waf1} and an inability to repress cyclin B.

As an initial approach to understand the apparent defect in the p53 pathway in MCF7 and HCT116 cells, we investigated the phosphorylation of p53 and the induction of an alternate p53 response gene. The HCT116 cells showed strong phosphorylation of p53 and strong induction of 14-3-3 σ immediately after damage. The MCF7 cells also showed phosphorylation of p53, albeit

much less than observed in the HCT116 cells, but they also showed strong induction of 14-3-3 σ . Hence, the markedly different kinetics of induction of 14-3-3 σ compared with p21^{waf1} in these two cell lines suggests that there is a selective defect in p53-mediated transactivation of p21^{waf1}.

It has been noted previously that the induction of p21^{waf1} may fail to occur in S phase after DNA damage (but still accumulate in G₁ and G₂) despite accumulation of p53 (26, 27). These reports contrast with the recognized ability of p21^{waf1} to bind to proliferating cell nuclear antigen and inhibit DNA synthesis (28) and that S-phase-arrested cells can exhibit elevated p21^{waf1} (29). As we find that many cell lines, both tumorigenic and nontumorigenic, are able to up-regulate p21^{waf1} during S-phase arrest, we believe this reflects the normal regulation of this protein. Hence, situations in which p21^{waf1} remains repressed in S-phase-arrested p53 wild-type cells appear to represent an abnormal situation.

p21^{waf1} is well known to be regulated at both transcription and post-translational levels. In many cell models, p53 has been shown to discriminate between induction of proapoptotic genes (e.g., PUMA, Noxa, and Bax) and cell cycle regulators (p21^{waf1}, GADD45, and 14-3-3 σ ; ref. 30), but a difference in regulation between two of the cell cycle regulators has not been reported previously. One potential means to discriminate between these two promoters arises from the ability of c-Myc to repress Miz, a transcription factor required for p53-mediated induction of p21^{waf1} (31, 32). In addition to p53-mediated induction of p21^{waf1}, there are numerous other pathways that have been implicated in p53-independent up-regulation of p21^{waf1} (33) or in stability of p21^{waf1} protein (34–37). Future research will investigate these alternate regulatory mechanisms.

It is worth considering the mechanism by which p21^{waf1} can suppress S-phase abrogation induced by UCN-01. DNA damage-induced activation of Chk1 leads to the phosphorylation and degradation of Cdc25A. In p53-defective cells, inhibition of Chk1 leads to rapid reaccumulation of Cdc25A and cell cycle progression. However, inhibition of Chk1 in p53 wild-type cells fails to induce the reaccumulation of Cdc25A, and this is a consequence of the p53 transactivation function rather than a direct p53-mediated repression (14). This was an unexpected observation as we had expected Cdc25A to reaccumulate on addition of UCN-01, but the cells to remain arrested in S phase because p21^{waf1} would inhibit Cdk2/cyclin E. We now show that DNA damage in the MCF10A cells suppresses both Cdc25A protein and mRNA. In contrast, the MCF10A/ Δ p21 cells failed to suppress Cdc25A mRNA such that UCN-01 was able to induce reaccumulation of Cdc25A protein and abrogate S-phase arrest. Accordingly, it appears that p21^{waf1} inhibits transcription of the Cdc25A mRNA. In this regard, there are two conflicting reports that have recently been published, one showing that damage-induced repression of Cdc25A mRNA is independent of p21^{waf1} (38), whereas the other shows suppression is mediated by direct binding of p21^{waf1} to the promoter of

Cdc25A (39). Our results are consistent with the second report. A possible reason for the discrepancy between these two reports is that the small interfering RNA used in the former research only partially down-regulated p21^{waf1} and this may have been inadequate to affect the Cdc25A promoter.

It is also important to note that the addition of UCN-01 to damaged HCT116 cells resulted in accumulation of p21^{waf1} in contrast to the suppression noted previously (10). We recognize that different clones of HCT116 cells can have notably different phenotypes, and we therefore repeated these experiments with HCT116 cells obtained from Dr. Vogelstein who was the source of the cells used in the former study. However, we obtained results identical to those shown in Fig. 4. Although the previous study damaged cells with γ -radiation rather than SN38, this appears unlikely to explain the discrepancy between these results as both experiments were done following activation of the checkpoint and removal of the insult. Although it was initially surprising that we observed cells entering mitosis despite the presence of high levels of p21^{waf1}, this is in agreement with other investigators who have made a similar observation in HCT116 cells (17).

Although p21^{waf1} appears critical for maintaining S-phase arrest on incubation with UCN-01, cyclin B is a critical regulator of mitosis. The ability of UCN-01 to induce mitosis correlated with expression of cyclin B. Furthermore, analysis of the MCF10A/ Δ p21 cells showed that the repression of cyclin B was independent of p21^{waf1} and is therefore consistent with the anticipated direct repression of cyclin B by p53. The inability of MCF7 and HCT116 cells to repress cyclin B when damaged explains their ability to undergo mitosis and shows that the apparent defect in p53 signaling in these cells extends to its repressor function.

The results of this study extend our previous results comparing the role of p53 in protecting cells from checkpoint abrogation by inhibitors of Chk1 (14, 19). Although we have assumed previously that p53 protected normal cells from UCN-01, we have confirmed that some p53 wild-type tumors are also resistant to UCN-01. However, we have identified several cell lines that are commonly considered to contain wild-type p53 but that are sensitive to UCN-01, and this is attributed to defects in p53-mediated transactivation of p21^{waf1} and repression of cyclin B. The importance of this observation for cancer therapy is that it extends the proportion of tumors that are candidates for treatment with Chk1 inhibitors. Future work will need to resolve the mechanism and determinants of response, so that patient tumors can be stratified and Chk1 inhibitors be administered to those patients whose tumor is predicted to be sensitive to the drug.

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References

1. Lau CC, Pardee AB. Mechanism by which caffeine potentiates lethality of nitrogen mustard. *Proc Natl Acad Sci U S A* 1982;79:2942–6.
2. Demarcq C, Bunch RT, Creswell D, Eastman A. The role of cell cycle progression in cisplatin-induced apoptosis in Chinese hamster ovary cells. *Cell Growth Differ* 1994;5:983–93.
3. Sarkaria JN, Busby EC, Tibbetts RS, et al. Inhibition of ATM and ATR kinase by the radiosensitizing agent, caffeine. *Cancer Res* 1999;59:4375–82.
4. Powell SN, DeFrank JS, Connell P, et al. Differential sensitivity of p53⁽⁻⁾ and p53⁽⁺⁾ cells to caffeine-induced radiosensitization and override of G₂ delay. *Cancer Res* 1995;55:1643–8.
5. Fan S, Smith ML, Rivet DJ, et al. Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. *Cancer Res* 1995;55:1649–54.
6. Bunch RT, Eastman A. Enhancement of cisplatin-induced cytotoxicity by 7-hydroxystaurosporine (UCN-01), a new G₂-checkpoint inhibitor. *Clin Cancer Res* 1996;2:791–7.
7. Wang Q, Fan S, Eastman A, Worland PJ, Sausville EA, O'Connor PM. UCN-01: a potent abrogator of G₂ checkpoint function in cancer cells with disrupted p53. *J Natl Cancer Inst* 1996;88:956–65.
8. Busby EC, Leistritz DF, Abraham RT, Karnitz LM, Sarkaria JN. The radiosensitizing agent 7-hydroxystaurosporine (UCN-01) inhibits the DNA damage checkpoint kinase hChk1. *Cancer Res* 2000;60:2108–12.
9. Graves PR, Yu L, Schwarz JK, et al. The Chk1 protein kinase and the Cdc25C regulatory pathways are targets of the anticancer agent UCN-01. *J Biol Chem* 2000;275:5600–5.
10. Yu Q, Rose JL, Zhang H, Takemura H, Kohn KW, Pommier Y. UCN-01 inhibits p53 up-regulation and abrogates γ -radiation-induced G₂-M checkpoint independently of p53 by targeting both of the checkpoint kinases, Chk2 and Chk1. *Cancer Res* 2002;62:5743–8.
11. Reinhardt HC, Aslanian AS, Lees JA, Yaffe MB. p53-Deficient cells rely on ATM- and ATR-mediated checkpoint signaling through the p38MAPK/MK2 pathway for survival after DNA damage. *Cancer Cell* 2007;11:175–89.
12. Fuse E, Tanii H, Kurata N, et al. Unpredicted clinical pharmacology of UCN-01 caused by specific binding to human α_1 -acid glycoprotein. *Cancer Res* 1998;58:3248–53.
13. Garber K. New checkpoint blockers begin human trials. *J Natl Cancer Inst* 2005;97:1026–8.
14. Levesque AA, Kohn EA, Bresnick E, Eastman A. Distinct roles for p53 transactivation and repression in preventing UCN-01-mediated abrogation of DNA damage-induced S and G₂ cell cycle checkpoints. *Oncogene* 2005;24:3786–96.
15. Husain A, Yan X-J, Rosales N, Aghajanian C, Schwartz GK, Spriggs DR. UCN-01 in ovary cancer cells: effective as a single agent and in combination with *cis*-diaminedichloroplatinum(II) independent of p53 status. *Clin Cancer Res* 1997;3:2089–97.
16. Hirose Y, Berger MS, Pieper RO. Abrogation of the Chk1-mediated G₂ checkpoint pathway potentiates temozolomide-induced toxicity in a p53-independent manner in human glioblastoma cells. *Cancer Res* 2001;61:5843–9.
17. Andreassen PR, Lacroix FB, Lohez OD, Margolis RL. Neither p21^{waf1} nor 14-3-3 σ prevents G₂ progression to mitotic catastrophe in human colon carcinoma cells after DNA damage, but p21^{waf1} induces stable G₁ arrest in resulting tetraploid cells. *Cancer Res* 2001;61:7660–8.
18. Wuerzberger-Davis SM, Chang P-Y, Berchtold C, Miyamoto S. Enhanced G₂-M arrest by nuclear factor- κ B-dependent p21^{waf1/cip1} induction. *Mol Cancer Res* 2005;3:345–53.
19. Kohn EA, Ruth ND, Brown MK, Livingstone M, Eastman A. Abrogation of the S phase DNA damage checkpoint results in S phase progression or premature mitosis depending on the concentration of UCN-01 and the kinetics of Cdc25C activation. *J Biol Chem* 2002;277:26553–64.
20. Gasparotto D, Maestro R, Piccinin S, et al. Overexpression of CDC25A and CDC25B in head and neck cancers. *Cancer Res* 1997;57:2366–8.
21. Giannini G, Ristori E, Cerignoli F, et al. Human MRE11 is inactivated in mismatch repair-deficient cancers. *EMBO Rep* 2002;3:248–54.
22. Tachibana KK, Gonzalez MA, Coleman N. Cell-cycle-dependent regulation of DNA replication and its relevance to cancer pathology. *J Pathol* 2005;205:123–9.

23. Harmes DC, Bresnick E, Lubin EA, et al. Positive and negative regulation of deltaN-p63 promoter activity by p53 and deltaN-p63- α contributes to differential regulation of p53 target genes. *Oncogene* 2003;22:7607–16.
24. Gudkov AV, Komarova EA. Prospective therapeutic applications of p53 inhibitors. *Biochem Biophys Res Commun* 2005;331:726–36.
25. Levesque AA, Eastman A. p53-Based cancer therapies: is defective p53 the Achilles heel of the tumor? *Carcinogenesis* 2007;28:13–20.
26. Gottifredi V, Shieh S, Taya Y, Prives C. p53 accumulates but is functionally impaired when DNA synthesis is blocked. *Proc Natl Acad Sci U S A* 2001;98:1036–41.
27. Delia D, Fontanella E, Ferrario C, Chessa L, Mizutani S. DNA damage-induced cell-cycle phase regulation of p53 and p21^{waf1} in normal and ATM-defective cells. *Oncogene* 2003;22:7866–9.
28. Waga S, Hannon GJ, Beach D, Stillman B. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* 1994;369:574–8.
29. Gottifredi V, McKinney K, Poyurovsky MV, Prives C. Decreased p21 levels are required for efficient restart of DNA synthesis after S phase block. *J Biol Chem* 2004;279:5802–10.
30. Meek DW. The p53 response to DNA damage. *DNA Repair (Amst)* 2004;3:1049–56.
31. Seoane J, Le H-V, Massague J. Myc suppression of the p21^{cip1} Cdk inhibitor influences the outcome of the p53 response to DNA damage. *Nature* 2002;419:729–34.
32. Herold S, Wanzel M, Beuger V, et al. Negative regulation of the mammalian UV response by Myc through association with Miz-1. *Mol Cell* 2002;10:509–21.
33. Gartel AL, Tyner AL. Transcriptional regulation of the p21^(WAF1/CIP1) gene. *Exp Cell Res* 1999;246:280–9.
34. Rossig L, Badorff C, Holzmann Y, Zeiher AM, Dimmeler S. Glycogen synthase kinase-3 couples AKT-dependent signaling to the regulation of p21^{Cip1} degradation. *J Biol Chem* 2002;277:9684–9.
35. Li Y, Dowbenko D, Lasky LA. AKT/PKB phosphorylation of p21^{Cip1/WAF1} enhances protein stability of p21^{Cip1/WAF1} and promotes cell survival. *J Biol Chem* 2002;277:11352–61.
36. Kim G-Y, Mercer SE, Ewton DZ, Yan Z, Jin K, Friedman E. The stress-activated protein kinases p38 α and JNK1 stabilize p21^{cip1} by phosphorylation. *J Biol Chem* 2002;277:29792–802.
37. Coleman ML, Densham RM, Croft DR, Olson MF. Stability of p21^{waf1Cip1} CDK inhibitor protein is responsive to RhoA-mediated regulation of the actin cytoskeleton. *Oncogene* 2006;25:2708–16.
38. Spurgers KB, Gold DL, Coombes KR, et al. Identification of cell cycle regulatory genes as principal targets of p53-mediated transcriptional repression. *J Biol Chem* 2006;281:25134–42.
39. Vigneron A, Cherier J, Barre B, Coquert O. The cell cycle inhibitor p21^{waf1} binds to the *myc* and *cdc25A* promoters upon DNA damage and induces transcriptional repression. *J Biol Chem* 2006;281:34742–50.