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UCN-01: a Potent Abrogator of G_2 Checkpoint Function in Cancer Cells With Disrupted p53

*Qizhi Wang, Saijun Fan, Alan Eastman, Peter J. Worland, Edward A. Sausville, Patrick M. O'Connor**

Background: **Arrest of the cell cycle in G_2 phase following DNA damage helps protect cell viability by allowing time for DNA repair before entry into mitosis (M phase). Abrogation of G_2 arrest sensitizes cells to the effects of DNA-damaging agents. UCN-01 (7-hydroxystaurosporine), a protein kinase C inhibitor that may block G_2 checkpoint regulation, has been reported to enhance the cytotoxicity of mitomycin C, a known DNA-damaging agent.** ***Purpose:*** **We studied the effect of UCN-01 on G_2 checkpoint control in human lymphoma CA46 cells, whose sensitivity to various DNA-damaging agents and G_2 response to DNA damage have been characterized. We also assessed the ability of UCN-01 to enhance the cytotoxicity of γ irradiation in CA46 cells and human colon carcinoma HT-29 cells, both of which are mutant for p53 function. The influence of p53 function on UCN-01-mediated abrogation of the G_2 checkpoint and enhancement of DNA-damaging agent cytotoxicity was studied in transfected human breast carcinoma MCF-7 cells that either expressed or did not express the human papillomavirus type-16 E6 protein. MCF-7 cells have normal p53 function, and the E6 protein binds p53 protein and promotes its destruction.** ***Methods:*** **The effect of UCN-01 on cell cycle arrest induced by γ irradiation was studied in CA46 cells and in transfected MCF-7 cells by use of flow cytometry. A histone H1 phosphorylation assay was employed to measure cyclin B1/Cdc2 kinase activity in extracts derived from irradiated and nonirradiated CA46 cells that had been either treated or not treated with UCN-01; the phosphorylation status of Cdc2 kinase protein in the same extracts was determined by use of western blotting. The effect of UCN-01 on the cytotoxicity of γ irradiation in CA46 and HT-29 cells was determined by use of MTT (thiazolyl blue) and clonogenic (colony-forming) assays, respectively; a clonogenic assay was also used to measure the effect of UCN-01 on the cytotoxicity of cisplatin in transfected and nontransfected MCF-7 cells.** ***Results:*** **G_2 arrest induced in CA46 cells by γ irradiation was inhibited by treatment with UCN-01 in a dose-dependent manner; arrest in G_2 was completely abrogated by exposure to 300 n*M* UCN-01. Biochemical markers indicative of the G_2/M transition, including the activation of cyclin B1/Cdc2 kinase and the suppression of Cdc2 threonine-14 and tyrosine-15 phosphorylation, were detected in irradiated cells treated with UCN-01. UCN-01 enhanced the cytotoxicity of γ irradiation in CA46 and HT-29 cells. MCF-7 cells with functional p53 protein were more resistant to G_2 checkpoint abrogation by UCN-01 than MCF-7 cells with disrupted p53 function. UCN-01 markedly enhanced the cell-killing activity of cisplatin in MCF-7 cells defective for p53 function.** ***Conclusions and Implications:*** **UCN-01 is a potent abrogator of G_2 checkpoint control in cancer cells with disrupted p53 function. UCN-01 might be capable of enhancing the effectiveness of DNA-damaging agents in the treatment of tumors with cells lacking normal p53 function.**
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Arrest of the cell cycle in G_2 phase following DNA damage is believed to promote cell viability by allowing time for DNA repair before entry into mitosis (M phase) (*1,2*). Consistent with this notion, agents that abrogate G_2 arrest (*3*) or mutations in genes that regulate the G_2 checkpoint (*4*) tend to sensitize cells to DNA-damaging agents. Agents that abrogate the G_2 checkpoint activated by DNA damage include the methylxanthines caffeine and pentoxifylline (*3,5,6*); the phosphatase inhibitors okadaic acid (*7*), fostriecin (*8,9*), and calyculin A (*10*); and certain protein kinase antagonists, such as staurosporine (*11*) and the aminopurines (*12*). The most potent checkpoint abrogators identified to date include staurosporine and the phosphatase inhibitors, which disable checkpoint control at nanomolar to low micromolar concentrations.

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See "Note" section following "References."

Staurosporine, originally isolated from the culture broth of *Streptomyces* (*13*), is a potent but relatively nonselective protein kinase antagonist that exhibits cytotoxicity toward cell lines in culture (*14*). Despite potent cytotoxic activity in vitro, staurosporine has not demonstrated antitumor activity in vivo (*15,16*), presumably because of its low therapeutic index. Furthermore, although staurosporine can abrogate G_2 arrest following DNA damage (*11,17*), its ability to do so is limited to a narrow concentration range, and staurosporine by itself at high concentrations can induce G_2 arrest in various cell lines (*18,19*). These effects of staurosporine have not encouraged its development for clinical studies. In contrast, UCN-01 (7-hydroxystaurosporine) is a more selective protein kinase antagonist than staurosporine in that it preferentially inhibits protein kinase C (*20,21*). UCN-01 also inhibits tumor cell growth in vitro at nanomolar concentrations (*14,15*), and it exhibits antitumor activity in vivo (*15,22*).

In an effort to define the relationship between protein kinase C inhibition and the growth inhibitory effects of UCN-01, Wang et al. (*23*) found that exposure of exponentially growing Jurkat cells to UCN-01 resulted in an inappropriate activation of the Cdc2 and Cdk2 kinases. Furthermore, activation of Cdc2 kinase was associated with a loss of cells from G_2 phase and an increase in the proportion of cells with either a G_1/S or hypodiploid DNA content. These events coincided with the induction of apoptosis. A possible explanation for such results is that UCN-01 promotes a bypass of the G_2 cell cycle checkpoint, thereby inducing premature chromosome condensation in interphase cells. The results also suggested that UCN-01 might be capable of blocking G_2 checkpoint regulation, since exposure to it caused a rapid decrease in the G_2-phase cell population. Interestingly, Akinaga et al. (*22*) had previously reported that UCN-01 and the DNA-damaging agent mitomycin C displayed synergistic toxic effects, both in vitro and in vivo, against human epidermoid A431 cancer cells. These findings suggest that UCN-01 might enhance the action of an agent known to activate the DNA-damage response pathway that normally operates in G_2 phase.

Entry into mitosis is regulated through formation and activation of the cyclin B1/Cdc2 kinase complex (*24*). Cyclin B1 synthesis starts in S phase, and, after complex formation, Cdc2 becomes phosphorylated at two residues associated with suppression of its activity (threonine-14 [T-14] and tyrosine-15 [Y-15]) and at one residue required for its activity (threonine-161 [T-161]) (*25*). Cdc2-inhibitory tyrosine phosphorylation can be mediated through the actions of the Wee1/Mik1 kinases (*26*); however, a recently discovered kinase that phosphorylates Cdc2 on both T-14 and Y-15 might also contribute to its inactivation (*27*). Removal of the inhibitory phosphates in human cells is carried out by the Cdc25C phosphatase (*28*). DNA damage-induced G_2 arrest is associated with the accumulation of relatively inactive, hyperphosphorylated cyclin B1/Cdc2 complexes [*see* (*1*) and references contained therein]. This response could be mediated through an increase in Cdc2-inhibitory kinase activity and/or a reduction in Cdc25C phosphatase activity. Pentoxifylline-induced G_2 checkpoint abrogation in human lymphoma cells is associated with a suppression of Cdc2-inhibitory phosphorylations (*5*). These findings suggest that agents that interfere with the G_2 checkpoint might operate by blocking the actions of Cdc2-inhibitory kinases (*1*); however, alternative mechanisms cannot be excluded.

To assess directly the effect of UCN-01 on G_2 checkpoint regulation, we employed cultured human lymphoma CA46 cells (mutant for p53 function), whose sensitivity to a variety of DNA-damaging agents (*29*) and whose G_2 checkpoint response to DNA damage have been characterized (*5,30*). We also investigated the effects of UCN-01 on the survival of CA46 cells and human colon carcinoma HT-29 cells (likewise mutant for p53 function) after exposure to ionizing radiation to determine whether alterations in cell cycle control processes would affect the cytotoxicity of this radiation. In addition, we performed other studies with human breast carcinoma MCF-7 cells (wild-type for p53 function) transfected with either a control plasmid (pCMV; yielding MCF-7/CMV cells) or a plasmid containing the human papillomavirus type-16 E6 gene (pCMV-E6; yielding MCF-7/E6 cells). The E6 gene product binds p53 protein and stimulates its destruction (*31,32*). Our purpose here was to determine whether p53 function influenced the ability of UCN-01 to enhance the cytotoxicity of DNA-damaging agents (e.g., cisplatin).

Materials and Methods

Chemicals and Reagents

Reagents for cell culture were purchased from Life Technologies, Inc. (Gibco BRL; Gaithersburg, MD). Unless otherwise specified, all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). UCN-01 (NSC-638850) was obtained from Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan).

Cell Culture

The human Burkitt's lymphoma cell line CA46 and the human colon carcinoma cell line HT-29 were obtained from the American Type Culture Collection (Rockville, MD). Derivatives of the human breast carcinoma cell line MCF-7 that are stably transfected with either an empty pCMV vector or the pCMV vector containing the human papillomavirus type-16 E6 gene have been described previously (*31*). All cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air in RPMI-1640 medium supplemented with heat-inactivated fetal bovine serum (15% vol/vol), penicillin G (50 U/mL), streptomycin (50 μg/mL), and L-glutamine (2 m*M*). The cell lines were maintained in exponential growth phase and were provided fresh medium every 2-3 days. Cell generation times ranged from 20 to 24 hours.

Flow Cytometry

Two to five million cells in 10 mL medium were pelleted by centrifugation at 500*g* for 5 minutes at room temperature, washed twice with ice-cold phosphate-buffered saline (PBS), and then fixed with 70% ice-cold ethanol. Before analysis by flow cytometry, the cells were washed with PBS, treated with RNase A (500 U/mL), and stained with propidium iodide (50 μg/mL). Cell cycle distribution assessments were performed using a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA); at least 15 000 cells per sample were analyzed.

Antibodies

A mouse monoclonal antiphosphotyrosine antibody (4G10 clone) was purchased from UBI (Lake Placid, NY). A polyclonal antibody raised against the carboxyl-terminus of Cdc2 was purchased from Life Technologies, Inc. The anti-cyclin B1 antibody has been described previously (*5*). Anti-rabbit and anti-mouse immunoglobulin G horseradish peroxidase-coupled antibodies were purchased from Bio-Rad Laboratories (Richmond, CA).

Irradiation and Drug Treatment

Exponentially growing CA46 cells were irradiated using a ^{137}Cs γ-ray source at a dose rate of 3.46 Gy/minute at room temperature. The cells were then incubated in the presence or absence of nocodazole (0.4 μg/mL) for 17 hours. UCN-01 was added to the cultures 6 hours after irradiation and/or the addition of nocodazole. Samples were processed for analysis 11 hours after the addition of UCN-01 or 17 hours after irradiation. In survival assays, cultures were exposed to UCN-01 for up to 24 hours following irradiation or treatment with cisplatin.

Immunoblotting, Immunoprecipitation, and Histone H1 Kinase Assays

CA46 cells were pelleted by centrifugation at 500*g* for 5 minutes at room temperature, washed once with 5 mL ice-cold PBS, and then lysed at a density of 1×10^8 cells/mL on ice for 10 minutes using a buffer that contained 10 m*M* sodium phosphate (pH 7.5), 100 m*M* NaCl, 5 m*M* sodium fluoride, 1.1 m*M* sodium vanadate, 1% Triton X-100, 0.5% sodium deoxycholate, 2 m*M* phenylmethylsulfonyl fluoride, and 20 μg/mL each of aprotinin and leupeptin. Cell lysates were clarified by centrifugation at 4 °C (15 minutes, 10 000*g*), and the resultant supernatants were used for protein analysis. For immunoblotting, 10-μg aliquots of total cell protein were subjected to electrophoresis in 10% polyacrylamide gels that contained 0.1% sodium dodecyl sulfate (SDS); the separated proteins were then transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) by electrophoresis at 500 V for 2.25 hours at 8 °C in a solution containing 10 m*M* 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11; 90% vol/vol) and methanol (10% vol/vol). Proteins were localized on the membranes by use of enhanced chemiluminescence procedures recommended by Amersham Life Science Inc. (Arlington Heights, IL). Immunoprecipitation reactions were carried out by incubating 600-μg aliquots of total cellular protein with 2 μg anti-Cdc2 antibody and 20-μL protein A–agarose beads (Oncogene Science, Inc., Cambridge, MA) at 4 °C for 3 hours; immunoprecipitation reaction tubes were rotated during the incubation period. The protein–agarose beads (and bound immune complexes) were then pelleted by centrifugation (5 seconds, 4 °C, 1000*g*), and the precipitated material was washed twice with kinase buffer (20 m*M* Tris–HCl [pH 7.6], 0.1 m*M* EGTA [ethylene glycol-bis{β-aminoethyl ether}*N*,*N*,*N*′,*N*′-tetraacetic acid], 1 m*M* dithiothreitol, and 10 m*M* $MgCl_2$). Histone H1 kinase assays were performed using a final adenosine triphosphate (ATP) concentration of 400 μ*M* (γ-^{32}P-labeled; specific activity, 200 cpm/pmol) as previously described (*23*).

Mitotic Index

Cells were pelleted by centrifugation (500*g*, 5 minutes, 4 °C), washed with ice-cold PBS, and resuspended in 0.5 mL cold, half-strength PBS for 10 minutes. They were then fixed with 6 mL 2% ethanol:acetic acid (3:1) for 30 minutes at 4 °C. Samples of the fixed cells were subsequently pelleted by centrifugation (500*g*, room temperature, 5 minutes), resuspended in 0.5 mL ethanol:acetic acid (3:1) for 10 minutes, and dropped onto glass slides; the slides were air-dried and stained with Giemsa. For each sample, at least 500 cells were randomly counted by use of light microscopy, and mitotic cells were scored by their lack of a nuclear membrane and evidence of chromosome condensation.

Cell Survival Assays

The effect of γ irradiation and UCN-01 treatment on the survival of CA46 cells was assessed using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium or thiazolyl blue) assay, essentially as described (*33*). Exponentially growing CA46 cells were irradiated and then incubated for 3 days with 100 n*M* UCN-01 at a density of 200 000 cells/mL in the wells of 96-well tissue culture plates (200-μL culture fluid per well) prior to the assessment of cell survival. The effect of γ irradiation and UCN-01 on the survival of human colon carcinoma HT-29 cells was assessed by use of a clonogenic (colony-forming) assay (*31*). Exponentially growing HT-29 cells were plated 24 hours prior to irradiation. After irradiation, the cells were incubated with UCN-01 (300 n*M*) for 24 hours, washed, and then incubated for 10-14 days before colonies (>50 cells) were counted. For MCF-7/CMV and MCF-7/E6 survival assays, cells were plated 24 hours prior to treatment with cisplatin for 1 hour and then incubated thereafter with 100 n*M* UCN-01 for 24 hours. After washing, the cells were incubated for 10-14 days before colony formation was assessed. The dose of UCN-01 used in the cell survival assays was the maximum dose that did not, by itself, induce cytotoxicity for the exposure times employed.

Results

Prevention of γ-Ray-Induced G_2 Arrest in Human Lymphoma CA46 Cells by Treatment With UCN-01

Exposure of asynchronously growing CA46 cells to 6.3 Gy of γ rays caused more than 88% of the cells to arrest in G_2 phase of the cell cycle (Fig. 1, panels designated 0 n*M* UCN-01). The addition of UCN-01 6 hours after irradiation, when most of the cells were in either S or G_2 phase (data not shown), resulted in a dose-dependent reduction in the size of the G_2-arrested population (Fig. 1, left panels). Concomitant with this change in the size of the G_2 population, the proportion of irradiated cells in G_1 and S phases was increased. This effect was apparent with 30 n*M* UCN-01; with 300 n*M* UCN-01, radiation-induced G_2 arrest was completely abolished (Fig. 1, left panels). Exposure of CA46 cells to 300 n*M* UCN-01 alone did not produce any dramatic alteration in cell cycle distribution or in cell death (as determined by trypan blue viability staining) within 24 hours of administration (data not shown). We confirmed that UCN-01 abrogated G_2 arrest, as opposed to inducing a G_1/S-phase block, in the first cell cycle by using the mitotic inhibitor nocodazole (*29*,*30*) in the experiments. Nocodazole prevented irradiated cells exposed to UCN-01 from progressing into the next cell cycle (Fig. 1, right panels). Thus, it is clear that the effect of UCN-01 is on the progression of cells from G_2 through M.

Association of Cdc2 Kinase Activation With UCN-01-Mediated Abrogation of G_2 Arrest

Arrest of cells in G_2 phase following DNA damage has been associated with a suppression of Cdc2 kinase activity (*5*,*30*,*34*). Consistent with these earlier observations, we found that Cdc2 kinase activity in γ-irradiated CA46 cells was suppressed when compared with the activity found in cells arrested in mitosis following treatment with nocodazole (Fig. 2, A). Inclusion of UCN-01 in the postirradiation medium resulted in the activation of Cdc2 kinase in a concentration-dependent manner (Fig. 2, B). This activation was observed most clearly when nocodazole was also included in the medium to prevent cells from exiting M phase (Fig. 2, B, filled bars). Indeed, we found that irradiated cells treated with 300 n*M* UCN-01 (a concentration that completely abolished G_2 arrest following γ irradiation) exhibited levels of Cdc2 kinase activity comparable to those seen when nonirradiated cells were incubated with nocodazole alone (Fig. 2, B). Furthermore, mitotic index measurements confirmed that irradiated cells treated with UCN-01 entered mitosis (data not shown). These results illustrate a close association between the ability of UCN-01 to abrogate γ-irradiation-induced G_2 arrest and the activation of Cdc2 kinase (comparison of Fig. 1 with Fig. 2, B).

Effects of UCN-01 and γ Irradiation on Cyclin B1 and Cdc2 Protein

Inactivation of Cdc2 following DNA damage has previously been linked to the presence of inhibitory phosphates on two

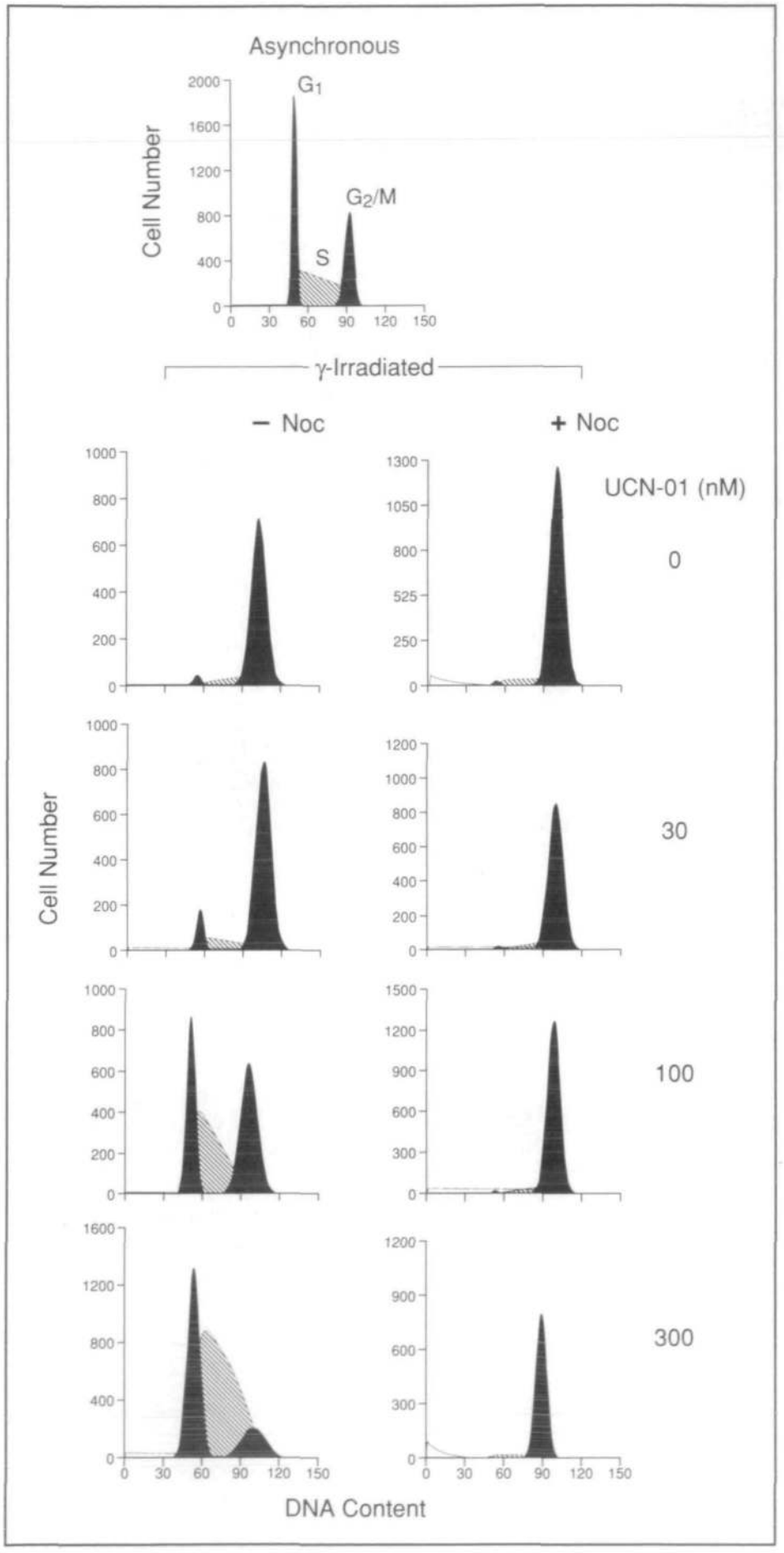

Fig. 1. UCN-01 abrogates radiation-induced G_2 arrest in CA46 cells. Asynchronously growing CA46 cells (top panel) were irradiated with 6.3 Gy of γ rays (lower panels) and then incubated in the absence (left panels) or the presence (right panels) of nocodazole (Noc = nocodazole, 0.4 μg/mL) for 17 hours. Six hours after irradiation, the cells were exposed to varying concentrations of UCN-01 (30, 100, or 300 n*M*), and, 11 hours later, they were harvested for analysis by flow cytometry. The flow cytometry plots shown are from an individual experiment that was conducted twice with similar results. The G_1, S, and G_2/M cell populations have been highlighted in the plot for the asynchronous control sample (top panel).

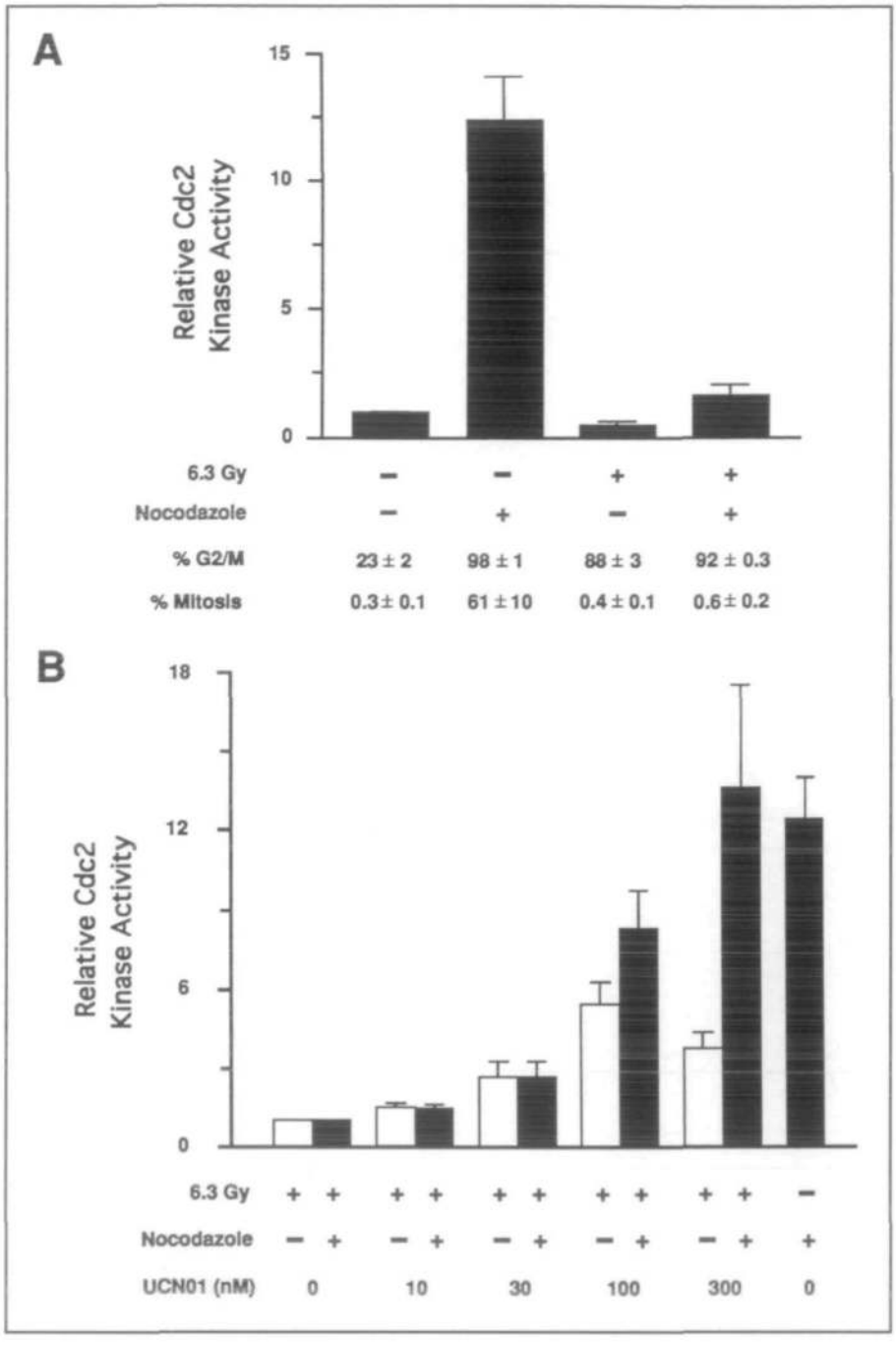

Fig. 2. Histone H1 kinase activity of Cdc2 in CA46 cells after irradiation and UCN-01 exposure. **A)** Comparison of Cdc2 kinase activity in extracts of exponentially growing, asynchronous cells compared with the activity in extracts of cells arrested in the G_2 phase of the cell cycle following γ irradiation (6.3 Gy; irradiated cells harvested 17 hours after irradiation) or in cells arrested in mitosis with nocodazole (0.4 μg/mL; cells harvested 17 hours after treatment with nocodazole ± irradiation). Cdc2 was immunoprecipitated using an anti-Cdc2 antibody and assayed as described in the "Materials and Methods" section. The relative histone H1 kinase activities of Cdc2 from asynchronously growing (lane 1), mitotic-arrested (lane 2), or G_2-arrested (lanes 3 and 4) cells are shown. Immunoprecipitated Cdc2 kinase activity from exponentially growing cells was 25-35 cpm ^{32}P incorporated/μg histone H1 per minute. For each sample, the percentage of cells in G_2/M and mitosis is indicated. **B)** Effect of UCN-01 on Cdc2 kinase activity in γ-irradiated CA46 cells. Irradiated cells were treated with UCN-01 in the absence (open bars) or the presence (closed bars) of nocodazole. Shown is the relative amount of Cdc2 kinase activity in each sample compared with the activity in cells that were not exposed to UCN-01. Also shown is the Cdc2 kinase activity in nonirradiated cells treated with nocodazole but not with UCN-01. Error bars shown in panels A and B indicate the standard errors of the means from at least four independent determinations.

residues near the adenosine triphosphate (ATP)-binding domain of the protein (T-14 and Y-15) (*24,25*). To determine whether UCN-01-induced activation of Cdc2 is due to a block in the accumulation of phosphorylated forms of this protein, we examined the Cdc2 present in the extracts used in the experiments shown in Fig. 2, B. Cdc2 obtained from total cell lysates of interphase cells migrates as three distinct bands in SDS–polyacrylamide gels (Fig. 3, second panel). The two uppermost bands contain Cdc2 that is phosphorylated on T-14 and/or Y-15 (*35-37*). We specifically assayed Cdc2–tyrosine phosphorylation by western blotting using an anti-phosphotyrosine antibody (Fig. 3, third panel). Irradiation increased the proportion of Cdc2 that was phosphorylated on T-14 and Y-15 (Fig. 3, compare lanes 1 and 3). The state of Cdc2 in G_2-arrested cells contrasted with the state of Cdc2 in cells arrested in mitosis. In mitotic-arrested cells, Cdc2 lacked T-14 and Y-15 phosphorylation (Fig. 3, compare lanes 1 and 2) and was fully active as a kinase (Fig. 2). When the T-14 and Y-15 sites on Cdc2 are dephosphorylated, the resulting active form of the protein has a slightly faster mobility in SDS–polyacrylamide gels (Fig. 3, lane 2). This faster mobility has been reported to be due to Cdc2 phosphorylation on an activating threonine residue, T-161 (*37,38*).

Addition of UCN-01 to irradiated CA46 cells caused a dose-dependent reduction of Cdc2 hyperphosphorylation, which was most evident when the cells were treated with 300 n*M* UCN-01 (Fig. 3, lanes 7 and 12). This dose of UCN-01 also completely abrogated G_2 arrest and fully activated the Cdc2 kinase in the damaged cells (as shown in Figs. 1-3). Complete abrogation of

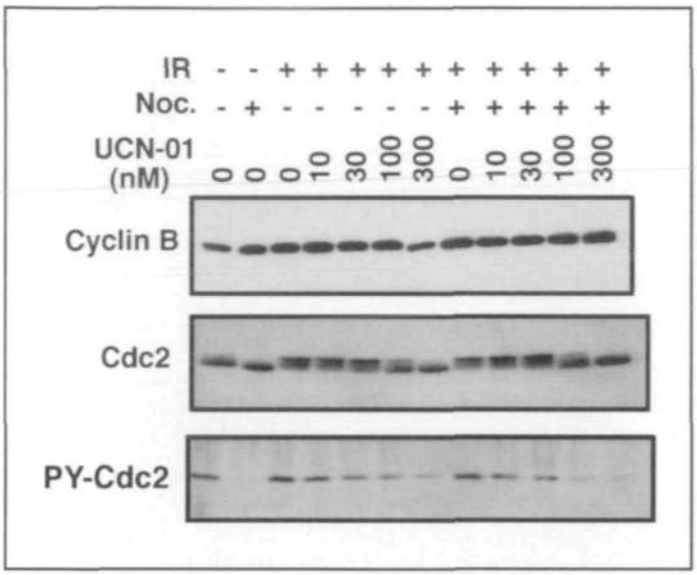

Fig. 3. Western blotting of Cdc2 and cyclin B1 proteins. CA46 cells were treated as described in the legend to Fig. 1. Protein from total cell lysates was separated in 10% polyacrylamide gels containing sodium dodecyl sulfate and then transferred to Immobilon-P membranes; the blotted proteins were probed with antibodies raised against cyclin B (upper panel blot) or Cdc2 (middle panel blot) (*see* "Materials and Methods" section for details). For analysis of Cdc2–tyrosine phosphorylation, protein was first immunoprecipitated from whole cell lysates (*see* the "Materials and Methods" section for details) and then subjected to western blotting; an anti-phosphotyrosine antibody was used to localize phosphorylated Cdc2 (py-Cdc2) on the blots (lower panel blot).

G_2 arrest and progression of CA46 cells into G_1 phase of the next cell cycle was associated with decreased cyclin B1 levels (Fig. 3, lane 7). This finding is consistent with the normal decline in cyclin B1 that occurs during cell division (*39*). In contrast, cells arrested in mitosis with nocodazole maintained cyclin B1 at increased levels (Fig. 3, lanes 2 and 12). These results suggested that UCN-01 abrogates G_2 arrest by preventing the accumulation of Cdc2 T-14 and Y-15 phosphorylations.

Cytotoxicity of γ Irradiation Increased by Treatment With UCN-01

We investigated the effects of UCN-01 on the cytotoxicity of γ irradiation in the p53-mutant cell lines CA46 and HT-29. Since CA46 cells do not form appreciable colonies, we monitored their survival using an MTT assay (*33*). We irradiated CA46 cells with different doses of γ rays and then incubated them in the presence of 100 n*M* UCN-01 for 3 days. This dose of UCN-01 was not cytotoxic by itself with the exposure time employed (data not shown). Inclusion of 100 n*M* UCN-01 in the postirradiation medium sensitized CA46 cells to γ rays (Fig. 4, A). The Do_{37} (dose required to inhibit cell survival by 37%) value for irradiated CA46 cells not incubated with UCN-01 was 2.8 Gy; the Do_{37} value for irradiated cells treated with UCN-01 was 1.65 Gy. Thus, UCN-01 potentiated the cytotoxicity of γ rays in CA46 cells by approximately 1.7-fold (Student's *t* test, two-sided $P<.05$).

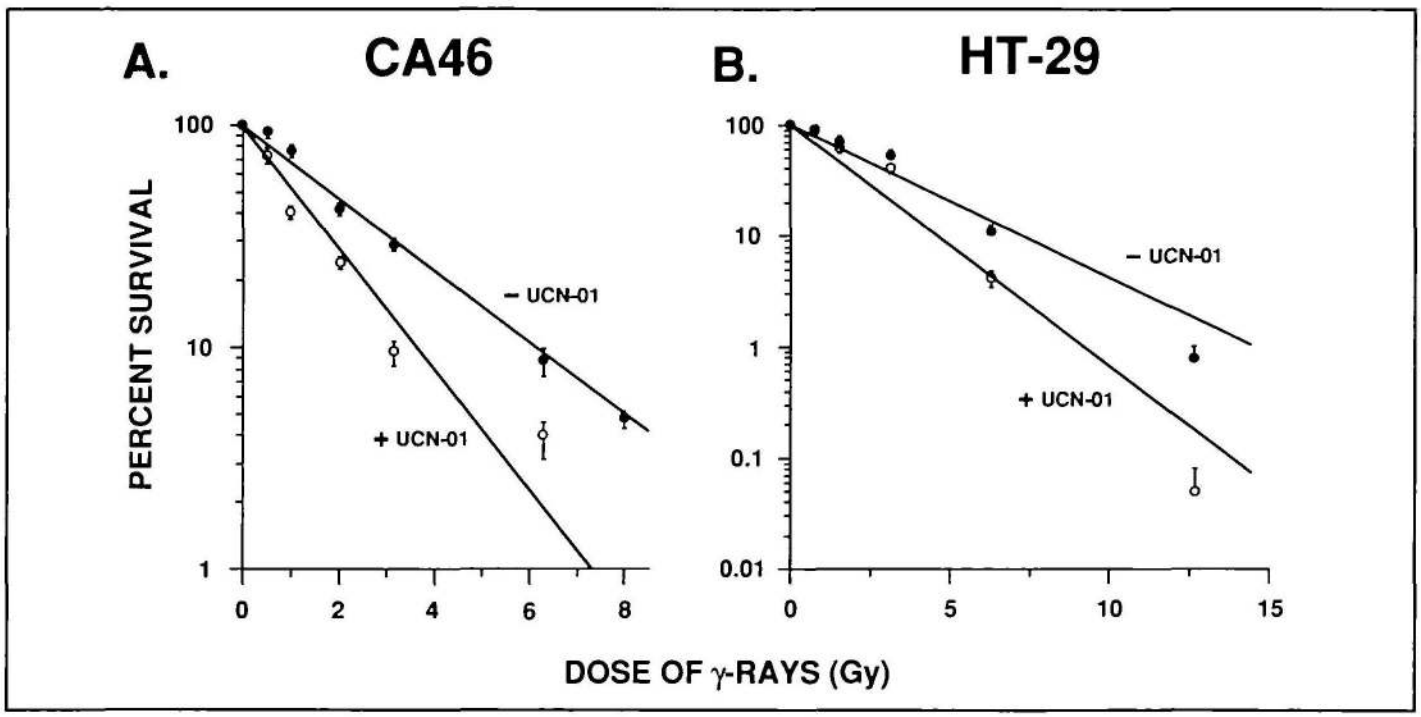

Fig. 4. UCN-01 increases the cytotoxicity of γ irradiation in human lymphoma CA46 cells and in human colon carcinoma HT-29 cells. A) Exponentially growing CA46 cells were irradiated and then cultured for 3 days in the presence or absence of UCN-01 (100 n*M*). Survival was then measured using the MTT (thiazolyl blue) assay (*33*). B) Exponentially growing HT-29 cells were plated 24 hours prior to irradiation. After irradiation, the cells were incubated in the presence or absence of UCN-01 (300 n*M*) for 24 hours, washed, and incubated for 10-14 days before colonies (>50 cells) were counted. The doses of UCN-01 used with CA46 and HT-29 cells were, by themselves, nontoxic (>90% survival, data not shown). Values shown are from a single experiment, representing the means and standard errors of the means from four samples. This experiment was repeated and yielded similar results. The linear fitted lines are shown. Do_{37} (the dose required to inhibit cell survival by 37%) = 2.8 for irradiated CA46 cells not incubated with UCN-01, and Do_{37} = 1.65 for irradiated CA46 cells treated with UCN-01 (ratio of Do_{37} values = 1.7). Do_{37} = 3.5 for irradiated HT-29 cells not incubated with UCN-01, and Do_{37} = 2 for irradiated HT-29 cells treated with UCN-01 (ratio of Do_{37} values = 1.75).

We also assessed the cytotoxicity of the combination of γ rays and UCN-01 in cells of the human colon carcinoma cell line HT-29. In these experiments, we monitored cell survival by use of a clonogenic assay (Fig. 4, B). Consistent with our results for CA46 cells, we found that HT-29 cells were more sensitive to γ irradiation in the presence of UCN-01. The Do_{37} value for irradiated HT-29 cells not treated with UCN-01 was 3.5 Gy; the Do_{37} value for irradiated HT-29 cells incubated with UCN-01 was 2 Gy (ratio of Do_{37} values = 1.75; Student's *t* test, two-sided $P<.05$). This degree of enhancement was similar to that previously reported for the combination of γ rays and the methylxanthine caffeine (*40,41*). Taken together, our results illustrate that UCN-01 can increase the cytotoxicity of γ irradiation in two diverse cancer cell lines that contain mutant p53 (also known as TP53) genes.

Preferential Enhancement of the Cytotoxicity of Cisplatin by Treatment With UCN-01 in MCF-7 Breast Cancer Cells With Disrupted p53 Function

Pentoxifylline, a caffeine derivative that abrogates the G_2 arrest induced by DNA damage, has been shown to enhance preferentially cisplatin-induced cytotoxicity in cells with disrupted p53 function (*28*). Therefore, we compared the effects of UCN-01 on the cytotoxicity of cisplatin in parental MCF-7 cells and in MCF-7 cells transfected with either control or human papillomavirus type-16 E6 gene-containing pCMV plasmids. MCF-7 cells are wild-type for p53, and their transfection with the papillomavirus E6 gene leads to a loss of p53 function, since the E6 polypeptide binds p53 protein and stimulates its destruction through the ubiquitin pathway (*31,32*). Exponentially growing cells were treated with cisplatin for 1 hour and then, in some cases, incubated thereafter with 100 n*M* UCN-01 for 24 hours.

The cisplatin sensitivity of control-transfected MCF-7 cells (MCF7/CMV, Do_{37} = 6 μ*M*) was similar to that observed for the parental cells (MCF-7, Do_{37} = 8.7 μ*M*) (Fig. 5) and, consistent with our previous observations (*31*), we found that MCF-7/E6 cells were more sensitive to cisplatin (Do_{37} = 2.6 μ*M*) than either the parental cells or the control transfectants (Fig. 5). For example, at the dose of cisplatin yielding 10% survival for MCF-7/CMV cells, the survival of MCF-7/E6 cells was 25-fold less. The dose modification factor for equivalent cisplatin killing in parental or control-transfected MCF-7 cells compared with MCF-7/E6 cells was approximately 2.8 (Student's *t* test, two-sided $P<.05$).

The addition of UCN-01 marginally increased the cytotoxicity of cisplatin in the parental and control-transfected MCF-7 cells (approximately 1.45-fold enhancement) (Fig. 5). However, the same treatment in MCF-7/E6 cells had a more pronounced effect on survival. For example, the Do_{37} value for cisplatin in the presence of 100 n*M* UCN-01 was 4.6 μ*M* for MCF-7/CMV cells and 1 μ*M* for MCF-7/E6 (Student's *t* test, two-sided $P<.05$). The extent to which MCF-7/E6 cells were preferentially sensitized to the combination of cisplatin and UCN-01 was similar to that previously reported for a combination of cisplatin and pentoxifylline (*28*).

Abrogation of G_2 Arrest in MCF-7 Cells With Disrupted p53 Function Following Treatment With UCN-01

The preferential activity of UCN-01 in MCF-7/E6 cells prompted us to determine whether these cells were more sensitive to UCN-01-induced G_2 checkpoint abrogation than the control transfectants. For simplicity, these studies were performed in exponentially growing cells exposed to ionizing radiation. The results are shown in Fig. 6. Incubation of MCF-7/CMV and MCF-7/E6 cells with the mitotic inhibitor nocodazole caused a marked accumulation of cells in G_2/M phase. This result confirmed that, during the course of the assay, both cell lines possessed the ability to progress through a nearly complete cell

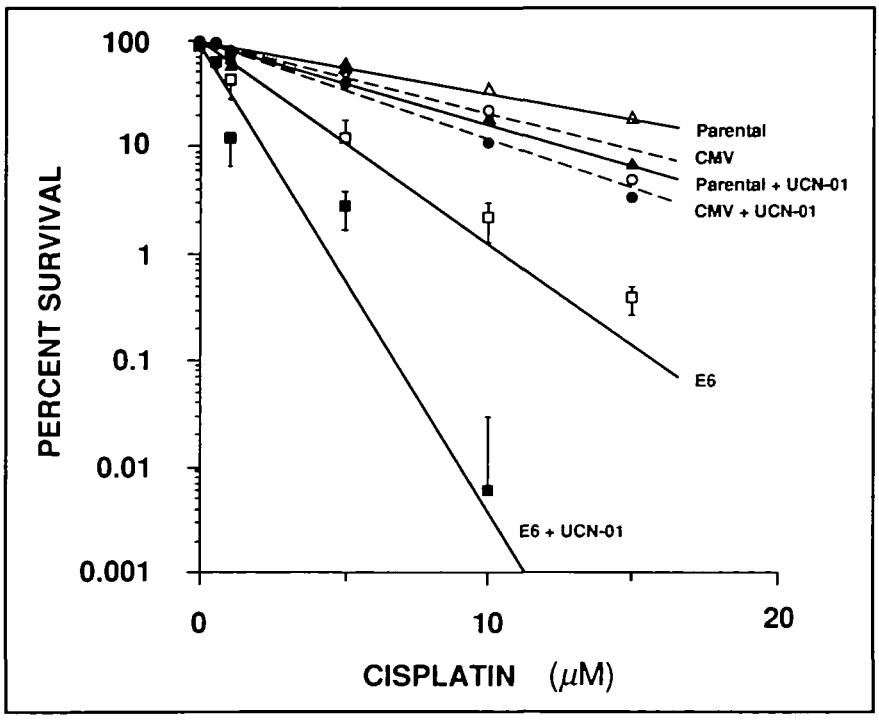

Fig. 5. UCN-01 preferentially increases cytotoxicity of cisplatin in MCF-7 cells with disrupted p53 function. Exponentially growing MCF-7/CMV and MCF-7/E6 cells were exposed to cisplatin for 1 hour, washed free of drug, and incubated for 24 hours in fresh medium that either contained or did not contain UCN-01 (100 n*M*). Following removal of UCN-01, the cells were incubated for 10-14 days before colonies (>50 cells) were counted. The survival fractions following treatment with cisplatin or cisplatin and UCN-01 are shown. Values shown for MCF7/E6 cells (E6) are from a single experiment, representing the means and standard errors of the means from four samples. This experiment was repeated and yielded similar results. Error bars have been omitted for the data obtained with MCF-7 (parental) and MCF7/CMV (CMV) cells for clarity of presentation. The linear fitted lines are shown. Do_{37} (dose required to inhibit cell survival by 37%) = 8.7 for cisplatin-treated parental MCF-7 cells, and Do_{37} = 5.4 for the combination of cisplatin and UCN-01 (ratio of Do_{37} values = 1.6). Do_{37} = 6 for cisplatin-treated control-transfected MCF-7 cells, and Do_{37} = 4.6 for the combination of cisplatin and UCN-01 (ratio of Do_{37} values = 1.3). Do_{37} = 2.6 for cisplatin-treated MCF-7/E6 cells, and Do_{37} = 1 for the combination of cisplatin and UCN-01 (ratio of Do_{37} values = 2.6).

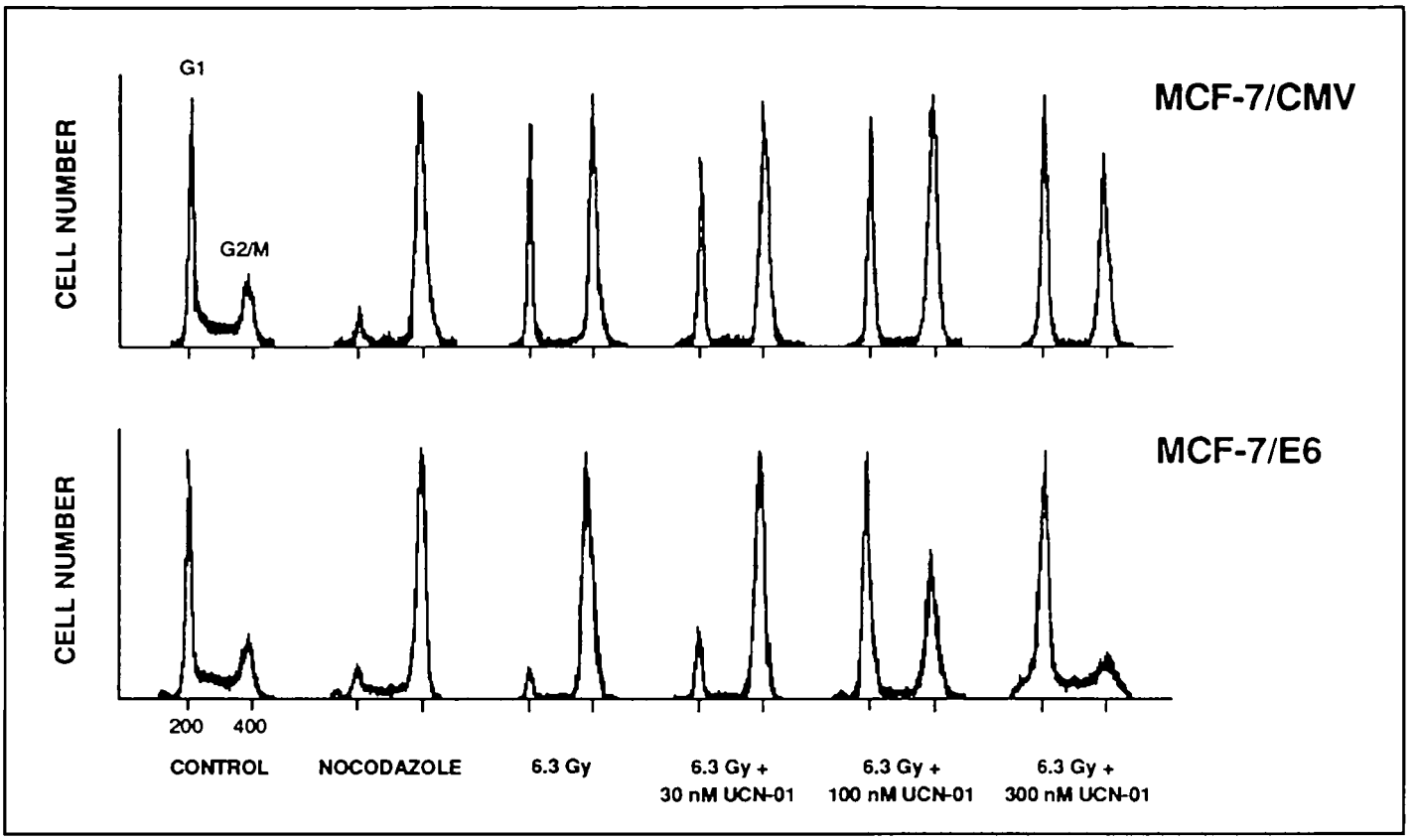

Fig. 6. UCN-01 preferentially abrogates γ-ray-induced G_2 arrest in MCF-7 cells with disrupted p53 function. Exponentially growing MCF-7/CMV and MCF-7/E6 cells were exposed to nocodazole (0.4 μg/mL) or γ rays (6.3 Gy); 16 hours later the cell-cycle distributions were analyzed by flow cytometry. In some cases, the irradiated cells were incubated after irradiation in medium that contained varying concentrations of UCN-01 (30, 100, or 300 n*M*). Shown is a representative data set from an individual experiment that was repeated twice. Cells in G_1 and G_2/M phases are highlighted in the upper panel.

cycle. Irradiation alone caused MCF-7/CMV cells to arrest in G_1 and G_2/M phases, whereas MCF-7/E6 cells, which lacked p53 function, arrested only in G_2/M phase (Fig. 6). Addition of nocodazole to the irradiated cultures did not significantly affect the DNA content patterns, confirming that the G_1-arrested population of MCF-7/CMV cells was arrested in G_1 phase of the first cell cycle (data not shown). Addition of UCN-01 following irradiation caused a dose-dependent abrogation of G_2 arrest in MCF-7/E6 cells. Treatment with 30 n*M* UCN-01 had a discernible effect in abrogating G_2 arrest; however, maximum G_2 checkpoint abrogation required a UCN-01 concentration of at least 100 n*M* (Fig. 6, lower panel). UCN-01 at less than 300 n*M* had a minimal effect on γ-ray-induced G_2 arrest in MCF-7/CMV cells. Our results are consistent with the notion that cells with disrupted p53 function are more sensitive to UCN-01-induced G_2 checkpoint abrogation than cells with an intact p53 control system.

Discussion

These experiments evaluated the effects of the protein kinase antagonist UCN-01 on γ-irradiation-induced G_2 arrest and survival of human cancer cell lines that differ in their p53 tumor suppressor status. We found that nanomolar concentrations of UCN-01 were sufficient to abrogate G_2 arrest in the p53-mutant cell line CA46. This result provided evidence that UCN-01 is one of the most potent G_2 checkpoint abrogators thus far described. Abrogation of the G_2 checkpoint was associated with an inhibition of Cdc2–tyrosine phosphorylation and activation of Cdc2 kinase activity. UCN-01 was much less active in abrogating the G_2 checkpoint in MCF-7 cells that had normal p53 function. A link between p53 status and sensitivity to G_2 checkpoint abrogation induced by UCN-01 was confirmed by disrupting p53 function in MCF-7 cells. Indeed, MCF-7 cells with disrupted p53 function were as sensitive to the effects of UCN-01 as the two p53-mutant cell lines tested (CA46 and HT-29) (compare Figs. 1 and 6). We also found that UCN-01 preferentially sensitized MCF-7/E6 cells to cisplatin-induced cytotoxicity, suggesting that UCN-01 might enhance the effectiveness and preference of DNA-damaging agents to cancer cells with disrupted p53 function.

Earlier work by Akinaga et al. (*22*) indicated that UCN-01 potentiated the cell-killing activity of DNA-damaging agents both in vitro and in vivo. Our findings support the notion that this result extends from the ability of UCN-01 to disable the G_2 checkpoint response to DNA damage. It has also recently been found that UCN-01 potentiates the cell-killing activity of cisplatin in CHO cells and that this enhanced cell killing is associated with abrogation of G_2 arrest following DNA damage (Bunch RT, Eastman A: manuscript submitted for publication). Our results are also concordant with those of Wang et al. (*23*), who found that treatment with UCN-01 induced premature ac-

tivation of the Cdc2 and Cdk2 kinases in T-cell lines and an associated loss of cells from the G_2/M portion of the cell cycle, as well as the appearance of cells with a hypodiploid DNA content. The CA46 cell line is particularly resistant to DNA damage-induced apoptosis (*29*), and this line has proven useful in investigating aspects of S and G_2 checkpoint control without the induction of apoptosis (*5,30*). By using CA46 cells, we were able to demonstrate that UCN-01 abrogated the cell-cycle arrest induced by ionizing radiation. The precise point at which UCN-01 interrupts the checkpoint response to DNA damage is presently under investigation. Since UCN-01 prevented the accumulation of inhibitory phosphates on Cdc2 following DNA damage, UCN-01 might either antagonize the Cdk-inhibitory kinases Wee1/Mik1 or enhance the activity of the Cdc25C phosphatase (*1,24,25*). In either case, Cdc2 would fail to accumulate inhibitory phosphates and would be inappropriately active in cells with DNA damage. We are presently conducting studies in FT210 cells, which have a temperature-sensitive Cdc2 (*42*), to confirm the requirement of Cdc2 activation in UCN-01-induced G_2 checkpoint abrogation.

The ability of UCN-01 to activate prematurely the Cdc2 kinase in cells with damaged DNA would limit the amount of DNA repair that could take place before cell division. As such, UCN-01 would be expected to sensitize cells to the lethal effects of DNA damage. In agreement with this possibility, we found that UCN-01, at concentrations without toxic effects as a single agent, increased the cytotoxicity of ionizing radiation in both CA46 and HT-29 cells (Fig. 4). Recently, we have also found that UCN-01 can abrogate the S-phase delay seen after treatment with DNA cross-linking agents, suggesting that disruption of this checkpoint might also contribute to enhanced cell killing (Fan S, Chang J, O'Connor PM: unpublished observations).

A number of compounds structurally related to UCN-01 have been studied in combination with DNA-damaging agents, especially staurosporine (*11,17*). The narrow dose range in which G_2 checkpoint abrogation by staurosporine can be achieved and the fact that, at higher concentrations, staurosporine induces G_2 arrest (*18,19*) complicates this agent's in vivo applicability. We have not observed G_2 arrest in CA46 cells treated with UCN-01 (Wang Q: unpublished observations). Furthermore, in contrast to staurosporine, UCN-01, at concentrations of 190 n*M* and below and for the exposure times used in our studies, is well tolerated in animals (Sausville EA: unpublished observations).

Fan et al. (*31*) recently showed that pentoxifylline preferentially sensitized MCF-7/E6 cells to cisplatin. Preferential sensitization was also reported by Powell et al. (*40*) and by Russell et al. (*41*) using a combination of caffeine and ionizing radiation. All three studies associated this activity with the preferential abrogation of the G_2 checkpoint in cells lacking p53 function. In comparison with pentoxifylline (*43*), UCN-01 is far more potent, with activity as a G_2 checkpoint abrogator at 30-300 n*M* (data from this study) versus millimolar concentrations for pentoxifylline (*5,40,41*). To study the relationship of this effect to p53 status, we used MCF-7 cells, which retain wild-type p53 function, and an MCF-7 derivative in which p53 function was disrupted by transfection with the human papillomavirus E6 gene. Our results with UCN-01 recalled those we obtained previously with pentoxifylline, in that UCN-01 was found to preferentially abrogate the G_2 checkpoint in MCF-7/E6 cells compared with MCF-7 control-transfected cells (*31*). A study (*29*) using a panel of Burkitt's lymphoma cell lines with either wild-type or mutant p53-gene status has also revealed the preferential activity of UCN-01 in abrogating γ-radiation-induced G_2 arrest in cells with defective p53 function (Fan S, Chang J, O'Connor PM: unpublished observations). Thus, taken together with results from other laboratories (*40,41*), it appears that cells with disrupted p53 function are more sensitive to some cell-cycle checkpoint abrogators. Such a vulnerability might be exploitable in an appropriately chosen combination chemotherapy or chemotherapy plus radiation protocol.

In summary, we found that UCN-01 is one of the most potent cell-cycle checkpoint abrogators thus far described, being active in cultured cells at nanomolar concentrations. Abrogation of the G_2 checkpoint was associated with inhibition of Cdc2 T-14 and Y-15 phosphorylations and activation of the Cdc2 kinase. These results suggest that UCN-01 might act by inhibiting the Wee1/Mik1 kinases that suppress Cdc2 activation and/or by activating the Cdc25C phosphatase that, in turn, activates Cdc2 (*27*). Studies with MCF-7 cells showed that cells with functional p53 protein were more resistant to the G_2 checkpoint-abrogating effects of UCN-01 and that disruption of p53 function in these cells sensitized them to UCN-01. Finally, UCN-01 preferentially enhanced the cell-killing activity of cisplatin in MCF-7 cells with defective p53 function. Our results suggest that UCN-01 might enhance the clinical antitumor effectiveness and preference of DNA-damaging agents to tumors with defective p53 function.

References

(*1*) O'Connor PM, Kohn KW. A fundamental role for cell cycle regulation in the chemosensitivity of cancer cells? Semin Cancer Biol 1992;3:409-16.

(*2*) Hartwell LH, Weinert TA. Checkpoints: controls that ensure the order of cell cycle events. Science 1989;246:629-34.

(*3*) Lau CC, Pardee AB. Mechanism by which caffeine potentiates lethality of nitrogen mustard. Proc Natl Acad Sci U S A 1982;79:2942-6.

(*4*) Weinert TA, Hartwell LH. The RAD9 gene controls the cell cycle response to DNA damage in *Saccharomyces cerevisiae*. Science 1988; 241:317-22.

(*5*) O'Connor PM, Ferris DK, Pagano M, Draetta G, Pines J, Hunter T, et al. G2 delay induced by nitrogen mustard in human cells affects cyclin A/cdk2 and cyclin B1/cdc2-kinase complexes differently. J Biol Chem 1993;268:8298-308.

(*6*) 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.

(*7*) Yamashita K, Yasuda H, Pines J, Yasumoto K, Nishitani H, Ohtsubo M, et al. Okadaic acid, a potent inhibitor of type 1 and type 2A protein phosphatases, activates cdc2/H1 kinase and transiently induces a premature mitosis-like state in BHK21 cells. EMBO J 1990;13:4331-8.

(*8*) Roberge M, Tudan C, Hung SM, Harder KW, Jirik FR, Anderson H. Antitumor drug fostriecin inhibits the mitotic entry checkpoint and protein phosphatases 1 and 2A. Cancer Res 1994;54:6115-21.

(*9*) Guo XW, Th'ng JP, Swank RA, Anderson HJ, Tudan C, Bradbury EM, et al. Chromosome condensation induced by fostriecin does not require p34cdc2 kinase activity and histone H1 hyperphosphorylation, but is associated with enhanced histone H2A and H3 phosphorylation. EMBO J 1995;14:976-85.

(*10*) Nakamura K, Antoku S. Enhancement of X-ray cell killing in cultured mammalian cells by the protein phosphatase inhibitor calyculin A. Cancer Res 1994;54:2088-90.

(*11*) Tam SW, Schlegel R. Staurosporine overrides checkpoints for mitotic onset in BHK cells. Cell Growth Differ 1992;3:811-7.

(*12*) Andreassen PR, Margolis RL. 2-Aminopurine overrides multiple cell cycle checkpoints in BHK cells. Proc Natl Acad Sci U S A 1992;89:2272-6.

(13) Tamaoki T, Nomoto H, Takahashi I, Kato Y, Morimota M, Tomita F. Staurosporine, a potent inhibitor of phospholipid/Ca++dependent protein kinase. Biochem Biophys Res Commun 1986;135:397-402.
(14) Tamaoki T, Nakano H. Potent and specific inhibitors of protein kinase C of microbial origin. Biotechnology 1990;8:732-5.
(15) Akinaga S, Gomi K, Morimoto M, Tamaoki T, Okabe M. Antitumor activity of UCN-01, a selective inhibitor of protein kinase C, in murine and human tumor models. Cancer Res 1991;51:4888-92.
(16) Meyer T, Regenass U, Fabbro D, Alteri E, Rosel J, Muller M, et al. A derivative of staurosporine (CGP 41 251) shows selectivity for protein kinase C inhibition and in vitro anti-proliferative as well as in vivo anti-tumor activity. Int J Cancer 1989;43:851-6.
(17) Bernhard EJ, Maity A, Muschel RJ, McKenna WG. Increased expression of cyclin B1 mRNA coincides with diminished G2-phase arrest in irradiated HeLa cells treated with staurosporine or caffeine. Radiat Res 1994;140:393-400.
(18) Abe K, Yoshida M, Usui T, Horinouchi S, Beppu T. Highly synchronized culture of fibroblasts from G2 block caused by staurosporine, a potent inhibitor of protein kinases. Exp Cell Res 1991;192:122-7.
(19) Crissman HA, Gadbois DM, Tobey RA, Bradbury EM. Transformed mammalian cells are deficient in kinase-mediated control of progression through the G1 phase of the cell cycle. Proc Natl Acad Sci U S A 1991;88:7580-4.
(20) Takahashi I, Kobayashi E, Asano K, Yoshida M, Nakano H. UCN-01, a selective inhibitor of protein kinase C from *Streptomyces*. J Antibiot 1987;40:1782-4.
(21) Takahashi I, Saitoh Y, Yoshida M, Sano H, Nakano H, Morimoto M, et al. UCN-01 and UCN-02, new selective inhibitors of protein kinase C. II. Purification, physico-chemical properties, structural determination and biological activities. J Antibiot 1989;42:571-6.
(22) Akinaga S, Nomura K, Gomi K, Okabe M. Enhancement of antitumor activity of mitomycin C in vitro and in vivo by UCN-01, a selective inhibitor of protein kinase C. Cancer Chemother Pharmacol 1993;32:183-9.
(23) Wang Q, Worland PJ, Clark JL, Carlson BA, Sausville EA. Apoptosis in 7-hydroxystaurosporine-treated T lymphoblasts correlates with activation of cyclin-dependent kinases 1 and 2. Cell Growth Differ 1995;6:927-36.
(24) Nurse P. Universal control mechanism regulating onset of M-phase. Nature 1990;344:503-8.
(25) Murray AW. Creative blocks: cell-cycle checkpoints and feedback controls [*see* comment citation in Medline]. Nature 1992;359:599-604.
(26) Lundgren K, Walworth N, Booher R, Dembski M, Kirschner M, Beach D. mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell 1991;64:111-22.
(27) Mueller PR, Coleman TR, Kumagai A, Dunphy WG. Myt1: a membrane-associated inhibitory kinase that phosphorylates Cdc2 on both threonine-14 and tyrosine-15. Science 1995;270:86-90.
(28) Hoffmann I, Clarke PC, Marcote MJ, Karsenti E, Draetta G. Phosphorylation and activation of human cdc25-C by cdc2—cyclin B and its involvement in the self-amplification of MPF at mitosis. EMBO J 1993;12:53-63.
(29) Fan S, el-Deiry WS, Bae I, Freeman J, Jondle D, Bhatia K, et al. p53 gene mutations are associated with decreased sensitivity of human lymphoma cells to DNA damaging agents. Cancer Res 1994;54:5824-30.
(30) O'Connor PM, Ferris DK, Hoffmann I, Jackman J, Draetta G, Kohn KW. Role of the cdc25C phosphatase in G2 arrest induced by nitrogen mustard. Proc Natl Acad Sci U S A 1994;91:9480-4.
(31) Fan S, Smith ML, Rivet DJ 2nd, Duba D, Zhan Q, Kohn KW, et al. Disruption of p53 function sensitizes breast cancer MCF-7 cells to cisplatin and pentoxifylline. Cancer Res 1995;55:1649-54.
(32) Kessis TD, Slebos RJ, Nelson WG, Kastan MB, Plunkett BS, Han SM, et al. Human papillomavirus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage. Proc Natl Acad Sci U S A 1993;90:3988-92.
(33) Carmichael J, DeGraff WG, Gazdar AF, Minna JD, Mitchell JB. Evaluation of a tetrazolium-based semiautomated colorimetric assay: assessment of chemosensitivity testing. Cancer Res 1987;47:936-42.
(34) Lock RB. Inhibition of p34cdc2 kinase activation, p34cdc2 tyrosine dephosphorylation, and mitotic progression in Chinese hamster ovary cells exposed to etoposide. Cancer Res 1992;52:1817-22.
(35) Morla AO, Draetta G, Beach D, Wang JY. Reversible tyrosine phosphorylation of cdc2: dephosphorylation accompanies activation during entry into mitosis. Cell 1989;58:193-203.
(36) Solomon MJ, Glotzer M, Lee TH, Philippe M, Kirschner MW. Cyclin activation of p34cdc2. Cell 1990;63:1013-24.
(37) Norbury C, Blow J, Nurse P. Regulatory phosphorylation of the p34cdc2 protein kinase in vertebrates. EMBO J 1991;10:3321-9.
(38) Lorca T, Labbe JC, Devault A, Fesquet D, Dapony JP, Cavadore JC, et al. Dephosphorylation of cdc2 on threonine 161 is required for cdc2 kinase inactivation and normal anaphase. EMBO J 1992;11:2381-90.
(39) Pines J, Hunter T. Isolation of a human cyclin B cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. Cell 1989;58:833-46.
(40) Powell SN, DeFrank JS, Connell P, Eogan M, Preffer F, Dombkowski D, et al. Differential sensitivity of p53(−) and p53(+) cells to caffeine-induced radiosensitization and override of G2 delay. Cancer Res 1995; 55:1643-8.
(41) Russell KJ, Wiens LW, Demers GW, Galloway DA, Plon SE, Groudine M. Abrogation of the G2 checkpoint results in differential radiosensitization of G1 checkpoint-deficient and G1 checkpoint-competent cells. Cancer Res 1995;55:1639-42.
(42) Th'ng JP, Wright PS, Hamaguchi J, Lee MG, Norbury CJ, Nurse P, et al. The FT210 cell line is a mouse G2 phase mutant with a temperature-sensitive CDC2 gene product. Cell 1990;63:311-24.
(43) Dezube BJ, Eder JP, Pardee AB. Phase I trial of escalating pentoxifylline dose with constant-infusion thiotepa. Cancer Res 1990;50:6806-10.

Note

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