A Novel Indolocarbazole, ICP-1, Abrogates DNA Damage-induced Cell Cycle Arrest and Enhances Cytotoxicity: Similarities and Differences to the Cell Cycle Checkpoint Abrogator UCN-01[1]

Alan Eastman,[2] Ethan A. Kohn, Mary Kay Brown, Joerg Rathman, Mark Livingstone, David H. Blank, and Gordon W. Gribble

Department of Pharmacology, Dartmouth Medical School, Hanover, New Hampshire 03755 [A. E., E. A. K., M. K. B.]; Department of Medicine, Dartmouth Hitchcock Medical Center, Lebanon, New Hampshire 03756 [J. R.]; Cell Signaling Technology, Inc., Beverly, Massachusetts 01915 [M. L.]; and Department of Chemistry, Dartmouth College, Hanover, New Hampshire 03755 [D. H. B., G. W. G.]

Abstract

DNA damaging agents such as cisplatin arrest cell cycle progression at either G_1, S, or G_2 phase, although the G_1 arrest is only seen in cells expressing the wild-type p53 tumor suppressor protein. We have reported that 7-hydroxystaurosporine (UCN-01) overcomes S and G_2 phase arrest and enhances the cytotoxicity of cisplatin. Abrogation of arrest appears to be selective for cells defective in p53 and therefore provides a potential, tumor-targeted therapy. Unfortunately, UCN-01 binds avidly to human plasma proteins, limiting access to the tumor. A screen of related indolocarbazoles identified analogues with both beneficial and undesirable properties. This led to a synthetic program to develop a novel analogue rationally designed to overcome the obstacles observed with the other analogues. We report the synthesis and analysis of a novel analogue, ICP-1. This analogue abrogated S and G_2 phase arrest and enhanced cytotoxicity induced by cisplatin only in p53 defective cells. ICP-1 also abrogated arrest and enhanced cell killing induced by the topoisomerase I inhibitor SN38. Analysis of proteins that regulate cell cycle arrest suggest both drugs inhibit checkpoint kinases Chk1 and/or Chk2. In contrast to UCN-01, checkpoint abrogation by ICP-1 was only slightly inhibited by human plasma. UCN-01 and ICP-1 differed significantly in other regards. UCN-01 potently enhanced the activity of 1-β-D-arabinofuranosylcytosine in both p53 wild-type and mutant cells, whereas ICP-1 was inactive in this combination. This property of UCN-01 was independent

of its ability to inhibit protein kinase C because more specific inhibitors of protein kinase C failed to enhance cell killing induced by 1-β-D-arabinofuranosylcytosine. High concentrations of UCN-01 also inhibit C-TAK1 that results in S phase-arrested cells directly entering mitosis, but this property was not observed with ICP-1. Hence, ICP-1 appears to be a more selective inhibitor of the S and G_2 cell cycle checkpoint than previously studied analogues and is worthy of study in preclinical tumor models.

Introduction

Much of the success in cancer chemotherapy has involved the use of drugs that damage the DNA of tumor cells (*e.g.,* cisplatin, mitomycin C, camptothecin, etoposide). However, the use of these drugs is limited by toxicity to the normal cells in the patient. DNA damaging agents cause arrest at either G_1, S, or G_2 phase of the cell cycle to permit time for DNA repair. The G_1 arrest is dependent upon the p53 tumor suppressor protein, hence p53-defective tumors arrest preferentially in S and G_2 phase. Caffeine can overcome this S and G_2 arrest (but not G_1 arrest), thereby driving cells through a lethal mitosis (1, 2). p53 wild-type cells will also arrest in S and G_2 phase, yet these cells are usually resistant to the effects of caffeine (3, 4). This suggests a therapeutic strategy that might selectively kill p53-defective cells while protecting the normal cells because they retain wild-type p53. Unfortunately, the required concentrations of caffeine cannot be tolerated by a patient. We recently reported that the indolocarbazole UCN-01[3] is 100,000-fold more potent than caffeine at abrogating S and G_2 arrest and also enhances cell killing selectively in p53 mutant cells (5, 6). Considering that >50% of tumors exhibit this defect, whereas normal cells do not, the combination of DNA damaging agents and UCN-01 has the potential to significantly improve therapeutic outcome.

UCN-01 has been shown to enhance the therapeutic activity of DNA damaging agents in animal models (7). In humans, UCN-01 has completed Phase I clinical trials as a single agent in both Japan and the United States. Unexpectedly, UCN-01 exhibited an extremely long half-life that resulted from avid binding to human plasma proteins (8, 9). The MTD in patients gave a mean plasma concentration of 36 μM, whereas <50 nM is required to abrogate cell cycle arrest *in vitro*. This was not predicted from the animal studies be-

[3] The abbreviations used are: UCN-01, 7-hydroxystaurosporine; MTD, maximum-tolerated dose; Cdk, cyclin-dependent kinase; araC, 1-β-D-arabinofuranosylcytosine; Chk, checkpoint kinase; PKC, protein kinase C.

cause UCN-01 did not bind to plasma proteins in these models. This raises concern as to whether UCN-01 can be combined effectively with DNA damaging agents in cancer patients.

We recently screened a panel of indolocarbazoles for their ability to abrogate cell cycle arrest (10). In addition to staurosporine and UCN-01, we identified one additional analogue, K252a, that also abrogated arrest, although this was restricted to abrogation of S-phase arrest. The reason K252a was unable to abrogate G_2 arrest was that, when used alone, it caused G_2 arrest. Indeed, staurosporine and another analogue, CGP41251, also caused G_2 arrest whereas UCN-01 did not. Considering that UCN-01 is the only one of these four analogues hydroxylated at the 7 position (Fig. 1), we surmised that this modification prevented G_2 arrest. These drugs all compete for the ATP binding site in protein kinases, and the crystal structure of staurosporine binding to Cdk2 suggested there was not enough room to accommodate a hydroxylated analogue in the ATP binding pocket (11). The amino acid sequence surrounding the ATP pocket in Cdk2 is conserved in the mitosis-inducing kinase Cdk1, and this possibly explains the inability of UCN-01 to cause G_2 arrest.

Additional studies with staurosporine demonstrated that the concentrations that abrogated cell cycle arrest were also cytotoxic to the cells (10). In contrast, UCN-01 abrogated arrest at noncytotoxic concentrations; a difference we also attributed to the 7-hydroxylation. These results show that slight modifications to the indolocarbazole structure can have profound effects on cell cycle perturbation and cytotoxicity. Finally, we confirmed that abrogation of arrest by both staurosporine and UCN-01 was prevented when cells were incubated in human plasma (see "Results"). However, K252a was still able to abrogate S-phase arrest in human plasma. This led to the proposal to modify the K252a structure with the hope that analogues would neither cause G_2 arrest nor bind plasma proteins. Here we report on results with the first analogue of this series, ICP-1, and show that it effectively abrogates S and G_2 arrest but has significantly different properties than UCN-01.

Materials and Methods

Drugs and Chemicals. Cisplatin in the form of Platinol (which contains saline and mannitol in water) was obtained from Bristol-Myers Squibb (Princeton, NJ). SN38 was provided by Dr. J. Patrick McGovren (Pharmacia Upjohn, Inc., Kalamazoo, MI). UCN-01 was provided by Dr. Edward Sausville (National Cancer Institute, Bethesda, MD). ICP-1 was synthesized as described below. These drugs were dissolved in DMSO. araC was purchased from Sigma (St. Louis, MO) and dissolved in water.

Cell Culture and Analysis. The breast cell lines used in this study were p53 wild-type, MCF10a, and p53 mutant MDA-MB-231 (American Type Culture Collection, Manassas, VA). The cells were maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml). In addition, the MCF10a cells were maintained in 8 μg/ml insulin, 20 ng/ml epidermal growth factor, and 500 ng/ml hydrocortisone.
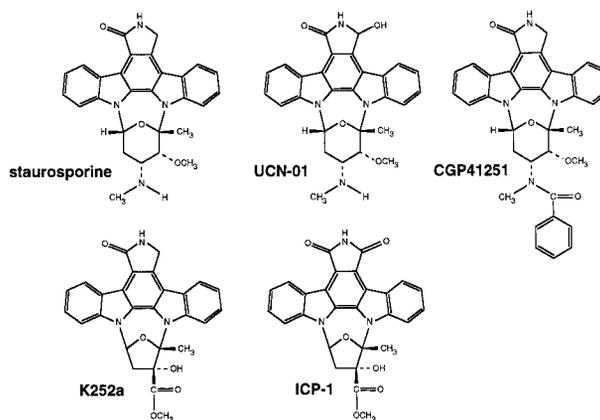

Fig. 1. Structure of the staurosporine analogues discussed in this paper.

Cells were incubated with cisplatin for 2 h; the drug was then removed, and the cells were incubated for an additional 22 h before addition of UCN-01 or ICP-1. Incubation of cells with SN38 was for 24 h, after which time the drug was removed, and the cells were incubated in fresh medium with or without the addition of UCN-01 or ICP-1. Incubations with araC were for 24 h concurrently with UCN-01 or ICP-1. Some incubations were performed in human plasma obtained using a heparinized needle and centrifuging at 2000 rpm for 10 min. Plasma was removed and stored in aliquots at $-20°C$ until use. All experiments reported here were performed with the same batch of human plasma to ensure a constant amount of plasma binding proteins.

Cell cycle analysis was performed according to a previously described procedure in which cells are harvested, fixed in ethanol, incubated with RNase, and stained with propidium iodide (2). DNA content was determined on a Becton Dickinson FACScan flow cytometer. Results are expressed as histograms because modeling programs, although good for calculating the number of cells in either G_1, S, or G_2, do not adequately reflect the progression through S phase (*i.e.,* discriminate early S, mid S, and late S phase). Furthermore, the binding of propidium iodide to DNA is dependent on chromatin structure (12), and extensive DNA breakage, as occurs upon incubation with SN38, can lead to an apparent increase in DNA content, particularly of G_2-arrested cells, and the modeling programs do not accommodate this situation (13).

Analysis of Cell Growth and Death. For analysis of cell growth, 500 MDA-MB-231 or 1000 MCF10a cells were plated in 100 μl in each well of a 96-well plate. The following day, drugs were added at the desired concentrations and with the required schedule to replicate wells (a minimum of 4/concentration). Drugs were removed, plates were rinsed, and then incubated for a total of 7 days from the initial drug addition. Inhibition of growth was then assessed on the basis of DNA content (14). Briefly, the media was removed, and attached cells were washed in 0.25× PBS, followed by the addition of 100 μl of H_2O. Cells were lysed by freeze/thawing the plates. Hoechst 33258 was added in high-salt buffer, incubated for 2 h, and fluorescence was measured on a CytoFluor II (PerSeptive Biosystems).

For analysis of cell viability, 75,000 cells were plated in 3 ml of medium and incubated with the required schedule of drugs. The cells were then incubated for 5 days from the initial drug addition. Detached and attached cells were harvested and incubated for 20 min in PBS containing 10 μM calcein-AM. Cells were then analyzed by flow cytometry. Viable cells were fluorescent, whereas dead cells were identified as nonfluorescent particles. Viability was also assessed in some experiments as cells that excluded trypan blue.

Immunoblotting. For immunoblot analysis, cells were rinsed with PBS, then lysed by direct addition of Laemmli sample buffer. Samples were immediately boiled for 5 min and stored at $-20°C$. Proteins were separated by SDS-PAGE (8%) and transferred to either nitrocellulose (Chk1/2) or polyvinylidene difluoride membranes (Cdc25C). Membranes were blocked with 5% nonfat milk in Tris-buffered saline, 0.1% Tween 20, and then probed with the appropriate antibody overnight at 4°C (Cdc25C, Neomarkers; phosphothreonine-48-Cdc25C, phosphothreonine-67-Cdc25C, phospho-Chk1, and phospho-Chk2, Cell Signaling). Membranes to be probed with the phosphothreonine-48-Cdc25C antibody were blocked and probed in 5% BSA rather than milk. Subsequently, membranes were washed in Tris-buffered saline, 0.1% Tween 20, and incubated with secondary antibody conjugated to horseradish peroxidase (Bio-Rad). Proteins were visualized by enhanced chemiluminescence (Amersham).

Synthesis of ICP-1. ICP-1 was synthesized by a modification of a published method (15) in which arcyriaflavin A was condensed with the appropriate dimethyl glycoside and camphorsulfonic acid and refluxed in 1,2-dichloroethane (76% yield on a small scale). Arcyriaflavin A was prepared according to the procedure of Harris *et al.* (16) in 54% yield from the palladium-mediated oxidative cyclization of the bisindolylmaleimide arcyriarubin A, which in turn was prepared in three steps from commercially available 3-indoleacetic acid. Thus, treating 3-indoleacetic acid with phosphorus pentachloride gave 3-indoleacetyl chloride (66% yield). This was treated with ammonia to give 3-indoleacetamide (53% yield). Base-catalyzed union of this amide with ethyl indolyl-3-glycoxylate according to the procedure of Faul *et al.* (17) yielded arcyriarubin A (50% yield). The ethyl indolyl-3-glycoxylate was synthesized from indole by sequential reaction with oxalyl chloride and then ethanol.

The dimethyl glycoside was synthesized according to a published method (15). Briefly, treatment of commercially available methyl acetoacetate with sodium hydride, followed by allyl bromide, gave the alkylated product methyl 2-allylacetoacetate in 84% yield. Oxidation of this compound as its *tert*-butyldimethylsilyl enol ether with *m*-chloroperbenzoic acid yielded methyl 2-allyl-2-hydroxyacetoacetate (49% yield for the two steps). Finally, ozonolysis of this material followed by treatment with methanol and camphorsulfonic acid gave the desired sugar as a mixture of diastereomers (45% yield).

The ICP-1 thus obtained from the union of the dimethyl glycoside and arcyriaflavin A was an orange solid, melting point 287°C. Analysis by high resolution mass spectroscopy gave *m/z* M$^+$ 481.1262 (the calculated value for $C_{27}H_{19}N_3O_6$ is 481.1274); MS *m/z* 481 (M$^+$, 95%), 446 (76%), 420 (66%), 378 (100%), 362 (51%), and 325 (52%). The base peak at *m/z* 378 probably results from cleavage of the sugar, resulting in loss of the fragment $C_4H_7O_3$. Both the proton and carbon nuclear magnetic resonance spectra of ICP-1 were consistent with the proposed structure.

Results

Abrogation of Cisplatin-induced S and G_2 Arrest by ICP-1. The cell models used in these studies were a p53 mutant breast cancer cell line, MDA-MB-231, and a p53 wild-type, immortalized breast cell line, MCF10a. These two cell lines were incubated with 20 μg/ml cisplatin for 2 h, a concentration determined to cause a significant accumulation of cells in S phase after 24 h (Fig. 2). We have previously shown that the addition of 50 nM UCN-01 22 h after removal of cisplatin forces the MDA-MB-231 cells through S to G_2 phase within 6 h and through a lethal mitosis within 24 h (10). In the current experiments, ICP-1 at various concentrations was added at 24 h (*i.e.*, 22 h after removal of cisplatin), and cells were analyzed for cell cycle perturbation after an additional 6 or 24 h. Within 6 h of addition of 250 nM ICP-1, most of the S phase-arrested MDA-MB-231 cells had been pushed through S phase with many appearing in G_2, whereas in the absence of ICP-1, a large proportion remained in S phase (Fig. 2*A*). After 24 h of ICP-1 (46 h after cisplatin), many of the cells had progressed through mitosis with only a few of the cells appearing in G_1, whereas many of the cells exhibited sub-G_1 DNA content. In this assay, 250 nM ICP-1 was as effective at abrogating arrest and enhancing cell death as our previous results with 50 nM UCN-01 (10). At these concentrations, neither UCN-01 nor ICP-1 perturbed cell cycle progression (data not shown).

Parallel experiments in MCF10a cells showed a partial accumulation in S phase 24 h after treatment with cisplatin (Fig. 2*B*). However, the addition of ICP-1 caused little change in the cell cycle profiles. These results are identical to those observed with UCN-01 (data not shown) and consistent with the hypothesis that p53 wild-type cells can be resistant to the effects of these checkpoint inhibitors.

Analysis of Proliferation and Cell Death Induced by Cisplatin plus ICP-1. The flow cytometry profiles described above show a marked increase in cells with sub-G_1 DNA content only for the p53 mutant cell line after addition of ICP-1. Microscopic analysis showed that most of these cells remained attached to the culture dish at this time and also retained viability as judged by exclusion of trypan blue, yet they exhibited a very heterogeneous size with most appearing far smaller than a normal cell. Various cytotoxicity assays were investigated but many were found to be inappropriate for quantifying cell death. For example, cisplatin induced a protracted G_2 arrest, yet most of these cells were not dead after 7 days. A clonogenic assay cannot discriminate dead cells from those exhibiting protracted arrest. An analysis of cell numbers showed up to 10-fold less proliferation than untreated cultures at some drug combinations, yet an assay of total protein (sulforhodamine B) did not reflect this because the arrested cells were much larger with much more protein. A similar inconsistency existed when using tetrazo-
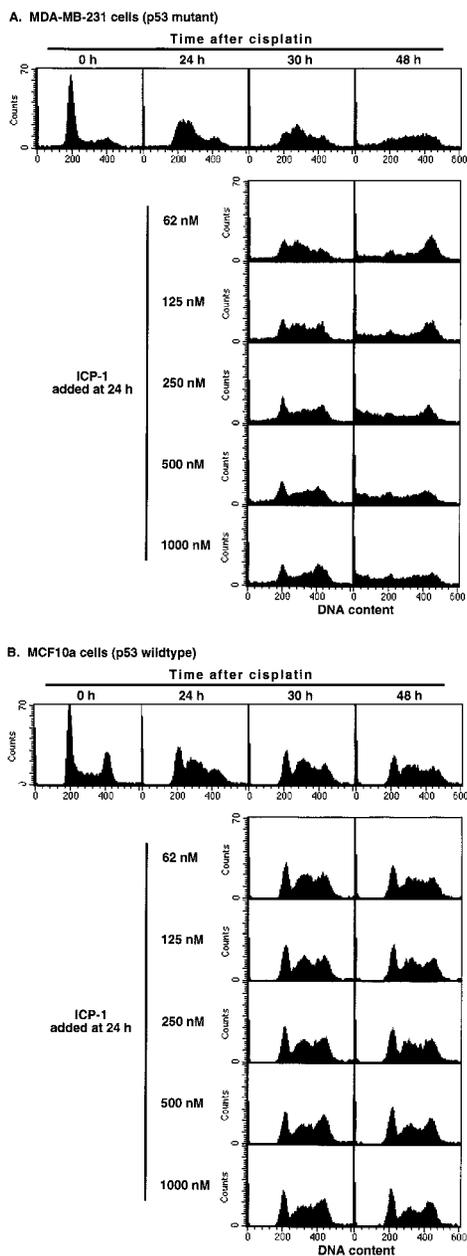
Fig. 2. The influence of ICP-1 on cell cycle perturbation induced by cisplatin in (A) MDA-MB-231 cells and (B) MCF10a cells. Cells were incubated with 20 μg/ml cisplatin for 2 h, the drug was removed, and the cells were incubated until harvest at 24, 30, or 48 h (*top panels*). At 24 h, ICP-1 was added at the indicated concentrations until harvest at 30 or 48 h. At harvest, cells were fixed and analyzed for cell cycle distribution by flow cytometry.

lium dyes that rely on mitochondrial dehydrogenases [*e.g.,* the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay].

In the experiments presented here, we have used two separate assays, one that measures cell proliferation, and the other that measures cell viability. We have found that cell proliferation can be rapidly and accurately assessed on the basis of DNA content (14). Cells are plated in a 96-well plate

at low density and assayed after 7 days of growth. Although cells arrested in G_2 will have 2-fold more DNA than those in G_1, this has little impact when compared with control cells that have undergone perhaps seven doublings during the time frame of the experiment. To assess the proportion of cells that remained viable, we modified the live/dead assay (Molecular Probes). For this analysis, cells were incubated with cisplatin for 2 h, then incubated for an additional 5 days. This time point was selected because preliminary experiments showed that the majority of cell death occurred around days 3 and 4; this was also the time frame of maximum death when UCN-01 and ICP-1 were added from 24–48 h. At longer times, many of the dead cells have disintegrated and cannot be scored (unfortunately, there is no single time point when all of the cells that die over the course of an experiment can be scored). Cells were then incubated with calcein-AM, which upon entry into live cells is de-esterified and fluoresces brightly. Dead cells are unable to de-esterify or retain this dye, so they do not fluoresce. The cells were then analyzed on a flow cytometer, and the number of live cells was quantified by fluorescence and compared with total cell number assessed by light scatter.

Using these assays, cells were scored for proliferation after a 2 h incubation with cisplatin (Fig. 3). Cisplatin at 5–10 μg/ml completely inhibited growth of both cell lines over the subsequent 7 days. However, these concentrations of cisplatin caused only a small decrease in cell viability. Concentrations of 80 μg/ml cisplatin were required to reduce cell viability by >50%. The addition of UCN-01 or ICP-1 from 24–48 h after cisplatin caused little additional inhibition of proliferation. However, both UCN-01 and ICP-1 markedly reduced viability in the MDA-MB-231 cells: >60% of the cells were dead at 10 μg/ml cisplatin. In contrast, the MCF10a cells were only slightly sensitized to cisplatin by UCN-01, and no reduction in viability was observed with ICP-1.

A simultaneous assessment of cell growth and death can be obtained on the basis of trypan blue exclusion. This assay is very labor intensive and hence not applicable to numerous drug concentrations as shown in Fig. 3. However, this assay was applied to a single concentration of cisplatin. Cells were incubated with or without 10 μg/ml cisplatin for 2 h, followed 24 h later by the addition of UCN-01 or ICP-1 (Fig. 3). Cisplatin inhibited growth in both cell lines, but there was no net loss of cells over 4 days. The addition of UCN-01 or ICP-1 to MDA-MB-231 cells reduced the cell number by about one log. There was a slight increase in viable cell number at the end of the 24-h incubation with UCN-01 or ICP-1 consistent with the cells being driven through mitosis, but these cells lost viability over the following days. In contrast, neither UCN-01 nor ICP-1 decreased viability of cisplatin-treated MCF10a cells. This experiment also shows that neither UCN-01 nor ICP-1 exhibit any toxicity in either cell line when used alone at these concentrations: the concentrations of UCN-01 and ICP-1 that inhibited proliferation by 50% were 100 nM and 1 μM in MDA-MB-231 cells, and 150 nM and 1.25 μM in MCF10a cells. Hence, ICP-1, like UCN-01, appears to selectively enhance cisplatin-induced cell killing only in the p53 mutant cell line.

A. MDA-MB-231 cells (p53 mutant)

Fig. 3. Inhibition of growth and viability induced by cisplatin in combination with either UCN-01 or ICP-1 in (*A*) MDA-MB-231 cells and (*B*) MCF10a cells. Cells were incubated with the indicated concentrations of cisplatin for 2 h, the drug was removed, and the cells were incubated until 24 h. A total of 50 nM UCN-01 or 250 nM ICP-1 was then added for the next 24 h. Cells were washed, and fresh media was added. *Left panels*: cells were harvested after 5 days and assayed for cell death (*right axis*) or harvested after 7 days and assayed for DNA content as a measure of proliferation (*left axis*). *Right panels*: cells were incubated with or without 10 μg/ml cisplatin for 2 h, then with or without UCN-01 or ICP-1. Viable cell number was assessed at the indicated times.

B. MCF10a cells (p53 wildtype)
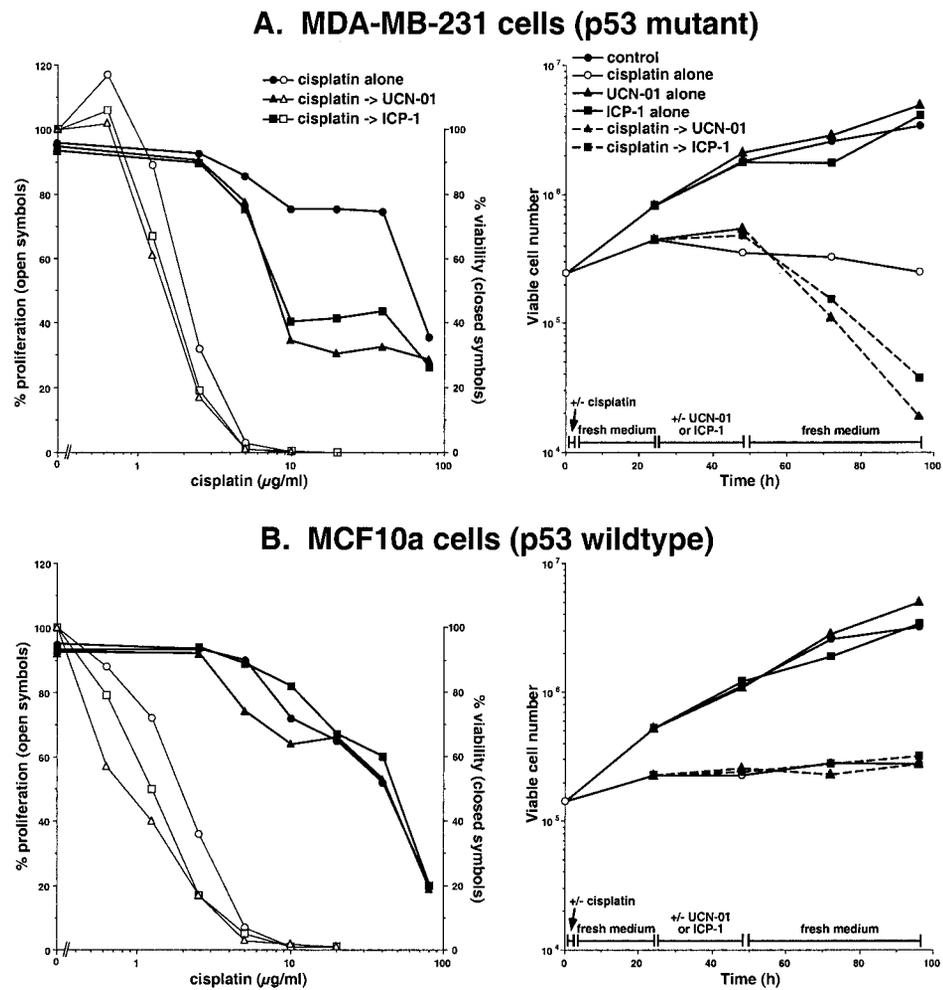
Abrogation of Cell Cycle Arrest Induced by SN38. A previous study has shown that UCN-01 can also abrogate S-phase arrest and enhance cytotoxicity induced by camptothecin (18). We have recently shown that UCN-01 can also abrogate S-phase arrest and enhance cytotoxicity induced by the topoisomerase I inhibitor SN38 (13). Therefore, we investigated the combination of ICP-1 and SN38. Both MDA-MB-231 and MCF10a cells were incubated with a range of concentrations of SN38 for 24 h. A G_2 arrest was observed at low concentrations, a mid-S-phase arrest at higher concentrations, and an early S-phase arrest at the highest concentrations (Fig. 4). SN38 was removed at 24 h, and the arrested cells progressed toward G_2 over the following 24 h. The MDA-MB-231 cells reached G_2 during this time frame, whereas the arrested MCF10a cells showed far less cell cycle progression. The addition of 50 nM UCN-01 or 250 nM ICP-1 at 24 h caused abrogation of G_2 arrest in MDA-MB-231 cells and induced a lethal mitosis of the majority of the cells $\geq$10 ng/ml SN38. We previously showed that the inclusion of nocodazole inhibited this lethal mitosis induced by UCN-01 (13), and similar protection was observed when nocodazole was added to the combination of SN38 and ICP-1 (data not shown). In contrast, MCF10a cells were unaffected by the addition of ICP-1.

There is an additional observation in these flow cytometry profiles that requires a comment. Cells incubated with SN38 frequently show a shift in the G_2 peak from 400 to ~480. This is because the amount of propidium iodide bound is not an absolute reflection of the amount of DNA in a cell but rather is dependent on the condensation of chromatin (12). In cells with extensive DNA breakage as induced by SN38, the chromatin is thought to relax and facilitate increased binding of the dye, and this is responsible for the apparent increase in DNA content. This effect is not seen with cisplatin-treated cells, consistent with the fact that cisplatin does not produce extensive breakage of DNA.

The experiments in Fig. 4 are worth an additional comment because it is clear that 10 ng/ml SN38 caused arrest of both cell lines in S phase at 24 h. Such an arrest was not as evident when the cells were incubated with cisplatin because of the additional G_1 arrest, particularly in the MCF10a cells (Fig. 2). DNA breaks induced by SN38 primarily occur during replication thereby avoiding the p53-dependent G_1 checkpoint. Hence, this model can be used to compare S-phase abrogation in p53 wild-type *versus* mutant cells. The cells were incubated for 24 h with 10 ng/ml SN38 to give a S-phase arrest, and then the efficacy of ICP-1 at abrogating this arrest was investigated. ICP-1 at 62 nM caused partial
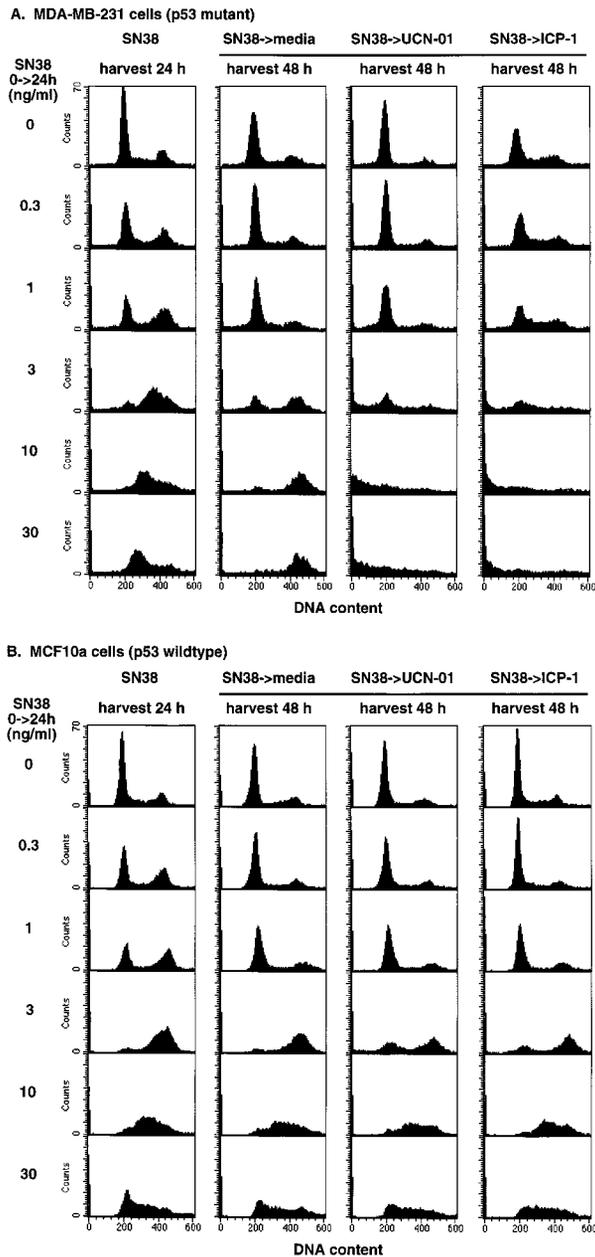
Fig. 4. The influence of UCN-01 and ICP-1 on cell cycle perturbation induced by SN38 in (*A*) MDA-MB-231 cells and (*B*) MCF10a cells. Cells were incubated with the indicated concentrations of SN38 for 24 h (*first panels*). The drug was removed, and cells were incubated for an additional 24 h in media (*second panels*), 50 nM UCN-01 (*third panels*), or 250 nM ICP-1 (*fourth panels*). Cells were harvested and analyzed for cell cycle distribution by flow cytometry.
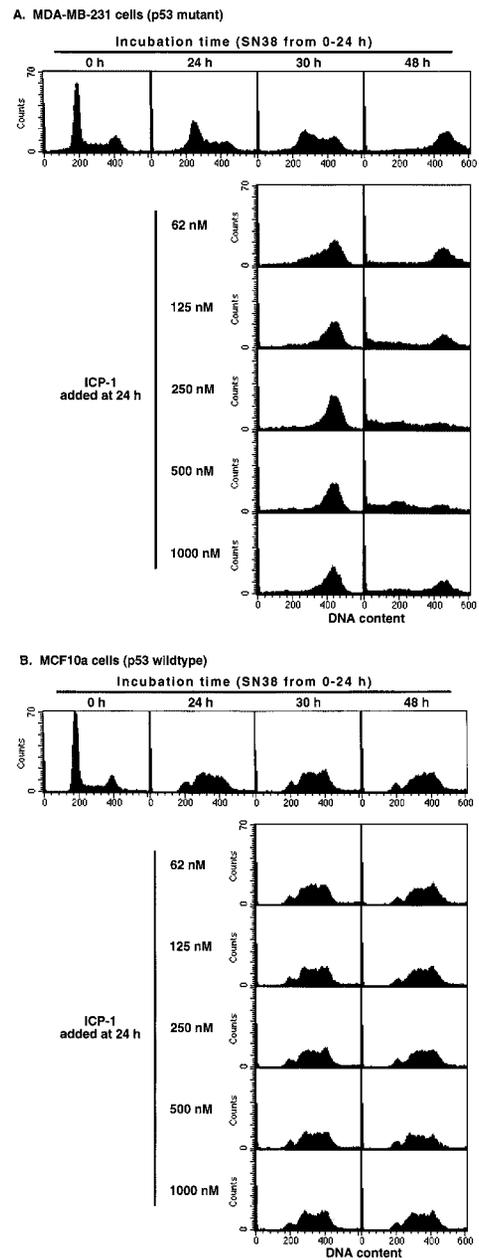
Fig. 5. The influence of ICP-1 on cell cycle perturbation induced by SN38 in (*A*) MDA-MB-231 cells and (*B*) MCF10a cells. Cells were incubated with 10 ng/ml SN38 for 24 h, the drug was removed, and the cells were immediately harvested or incubated until harvest at 30 or 48 h (*top panels*). At 24 h, ICP-1 was added at the indicated concentrations until harvest at 30 or 48 h. At harvest, cells were fixed and analyzed for cell cycle distribution by flow cytometry.

abrogation of S-phase arrest in MDA-MB-231 cells over the following 6 h (Fig. 5). ICP-1 at 250 nM caused complete abrogation of S-phase arrest within 6 h and almost complete abrogation of G_2 within 24 h. This can be compared with the efficacy of UCN-01 in this model in which abrogation of S-phase arrest occurs at 2 nM and abrogation of G_2 arrest occurs at 7.5 nM (13). In contrast, neither ICP-1 (Fig. 5*B*) nor UCN-01 (13) up to 1 μM abrogated arrest in the MCF10a cells.

Analysis of Proliferation and Cell Death Induced by SN38 plus ICP-1. Inhibition of cell growth was measured with the DNA assay as described above. SN38 was equally effective at inhibiting cell growth in the two cell lines (Fig. 6). However, concentrations that inhibited growth (>3 ng/ml) were more effective at decreasing viability in the MDA-MB-231 cells. This correlates with the observation that these cells continued to replicate slowly after removal of SN38, whereas

Fig. 6. Inhibition of growth and viability induced by SN38 in combination with either UCN-01 or ICP-1 in (*A*) MDA-MB-231 cells and (*B*) MCF10a cells. Cells were incubated with the indicated concentrations of SN38 for 24 h, followed by either 50 nM UCN-01 or 250 nM ICP-1 for the next 24 h. Cells were washed, and fresh media was added. *Left panels*: cells were harvested after 5 days and assayed for cell death (*right axis*) or harvested after 7 days and assayed for DNA content as a measure of proliferation (*left axis*). *Right panels*: cells were incubated with or without 5 ng/ml SN38 for 24 h, then with or without UCN-01 or ICP-1. Viable cell number was assessed at the indicated times.
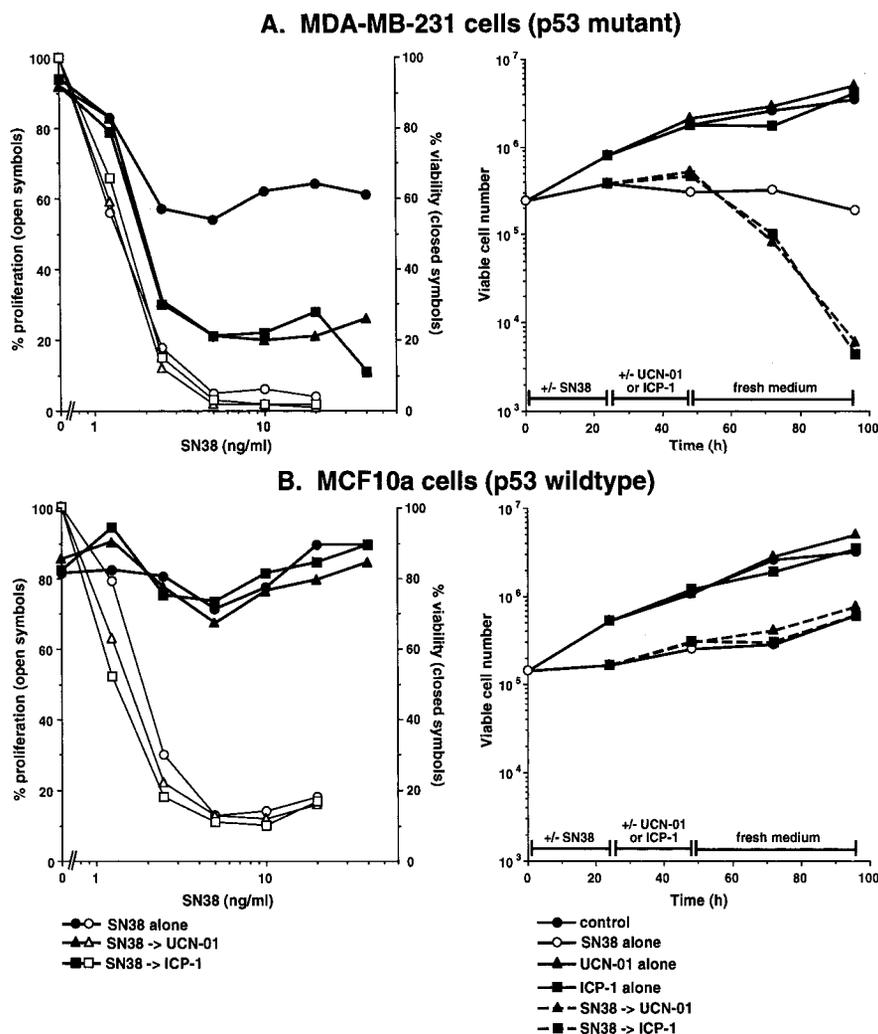
the MCF10a cells progressed little (between 24 and 48 h; Figs. 4 and 5). There is a suggestion that higher concentrations of SN38 were less toxic to MCF10a cells than the intermediate concentrations, and this might be explained by the higher concentrations arresting cells earlier in the cell cycle and thereby protecting them. We have not determined whether these concentrations of SN38 kill the MCF10a cells at a later time but we consider it likely.

The addition of UCN-01 or ICP-1 to SN38-treated cells caused little further inhibition of growth but significantly decreased viability in the MDA-MB-231 cells (Fig. 6). Again, the MCF10a cells appeared completely refractory to this effect of the checkpoint inhibitors. This analysis was extended using trypan blue exclusion after cells had been incubated with 5 ng/ml SN38. The results show that the addition of either UCN-01 or ICP-1 caused a marked decrease in viable MDA-MB-231 cells but had no effect in the MCF-10a cells. Hence, as with cisplatin, it appears that the cytotoxicity of SN38 can be enhanced by UCN-01 and ICP-1 selectively in the p53 mutant cell line.

Comparison of UCN-01 and ICP-1 in Combination with araC. It has previously been shown that UCN-01 can enhance the cytotoxicity of nucleoside analogues such as gemcitabine and araC and that this can occur without cell cycle progression (19). In those studies, UCN-01 was added without removal of the nucleoside analogue. Here, we investigated the effects of a concurrent incubation of araC and the checkpoint inhibitors. To assess the effect of drug combinations on proliferation, cells were incubated with drugs for 24 h, then rinsed and incubated for an additional 6 days before assessment of DNA content/well. The addition of 50 nM UCN-01 caused a marked increase in sensitivity to araC in both cell lines; 15 nM UCN-01 caused partial sensitization (Fig. 7*A*). In contrast, ICP-1 had no impact on the sensitivity of cells to araC.

In seeking an explanation for this difference between the action of UCN-01 and ICP-1, we analyzed cell cycle perturbation after a 24-h incubation of the drug combinations. When MDA-MB-231 cells were incubated with araC alone, a G_2 arrest was evident at 2–3 μM and a S-phase arrest at 4–5 μM (Fig. 7*B*). The addition of UCN-01 caused arrest earlier in
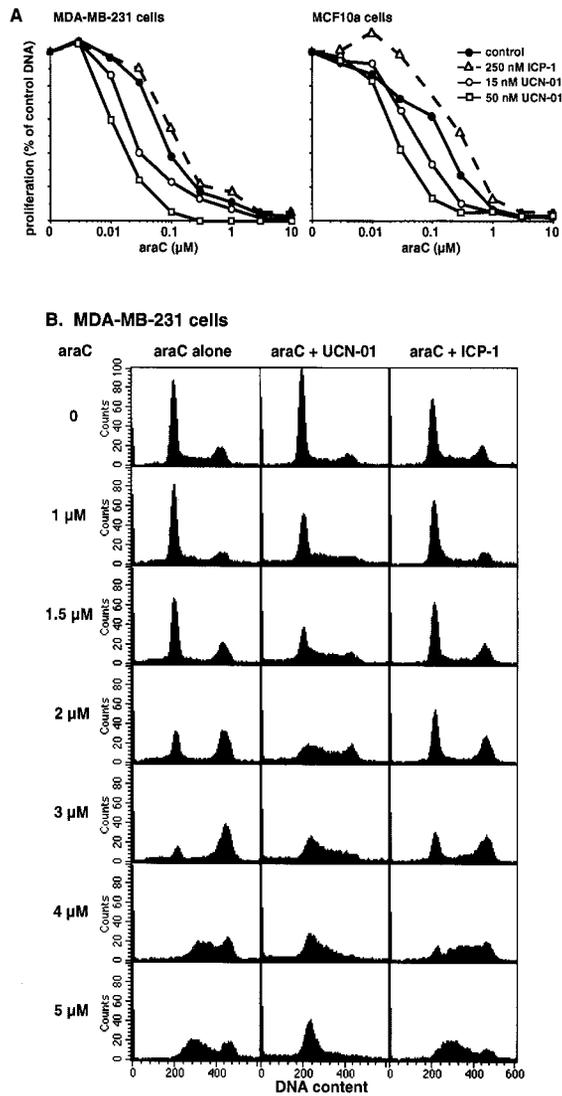
Fig. 7. The influence of UCN-01 and ICP-1 on the sensitivity of cells to araC. *A*, MDA-MB-231 cells (*left*) or MCF10a cells (*right*) were incubated for 24 h with the indicated concentrations of araC either alone or in combination with 15 nM UCN-01, 50 nM UCN-01, or 250 nM ICP-1. The drugs were removed, and the cells were allowed to grow for another 6 days before harvest and analysis of DNA content as a measure of proliferation. *B*, MDA-MB-231 cells treated as in *A* were harvested at 24 h, fixed, and analyzed for cell cycle distribution by flow cytometry.

the cell cycle at all concentrations of araC. For example, there was a clear S-phase arrest at 2 μM araC and a G_1 arrest at 4–5 μM. Hence, it appears that UCN-01 is enhancing the damage induced by araC, possibly by increasing its incorporation into DNA. This could occur by increasing the activation or decreasing the inactivation of araC. In contrast, ICP-1 had little effect on the araC-mediated cell cycle perturbation. Similar results were observed with MCF10a cells (data not shown).

UCN-01 was originally reported to be a PKC inhibitor (20, 21). Therefore, we determined whether this sensitization of cells to araC would occur upon incubation with other PKC inhibitors. We observed no sensitization with either

GF109203X up to 5 μM or chelerythrine chloride up to 2 μM (data not shown). Accordingly, this sensitization by UCN-01 appears to relate to a currently undefined target.

Influence of Human Plasma on Abrogation of Cell Cycle Arrest. Considering that the clinical efficacy of UCN-01 may be compromised by its binding to human plasma proteins, we established a bioassay to assess the binding capacity of these analogues. In all of the above analyses, cells were incubated in medium with 10% fetal bovine serum. For comparison, UCN-01 or ICP-1 was added in 5% human plasma plus 5% bovine serum. Results are shown for human plasma obtained from a single individual on a single occasion to ensure no variation in the level of plasma-binding proteins. Although we have used 50 nM UCN-01 for most of the comparisons in this paper, abrogation of S and G_2 arrest in bovine serum can be induced by <10 nM UCN-01 (13). In the presence of human plasma, abrogation of S-phase arrest was not observed until 500 nM UCN-01, and significant abrogation of G_2 arrest required 1 μM (Fig. 8). Hence, 100-fold more drug was required in human plasma for abrogation of arrest.

In the case of ICP-1, 250 nM caused complete abrogation of S and G_2 arrest in bovine serum (Fig. 5*A*), whereas slight abrogation of S phase was still observed in human plasma at this concentration (Fig. 8). Partial abrogation of G_2 arrest occurred at 500 nM ICP-1 while complete abrogation required 1 μM ICP-1. Hence, about 4-fold more drug is required for abrogation of arrest. We tested the possibility that bovine serum was able to bind ICP-1, but in serum-free medium, 250 nM ICP-1 was still required for abrogation of S-phase arrest (data not shown). Similarly, 7.5 nM UCN-01 still abrogated arrested in serum-free medium. Hence, neither drug appear to bind to bovine serum. Accordingly, it appears that ICP-1 is inhibited substantially less by the presence of human plasma proteins.

Mechanism of Action of ICP-1. The ability of UCN-01 to abrogate cell cycle arrest is thought to be mediated via its inhibition of Chk1 (22) and possibly Chk2 (23), both of which suppress the mitosis-inducing phosphatase Cdc25C. Alternately, caffeine has been shown to abrogate arrest through inhibition of ataxiatelangiectasia-mutated (ATM) and/or ataxiatelangiectasia-related (ATR), which are required for activation of Chk1/2 (24, 25). To investigate these possible targets, we determined whether ICP-1 inhibited the activation of Chk1 and Chk2, which would suggest ICP-1 acts upstream, or whether it inhibited phosphorylation of Cdc25C, like UCN-01, thereby suggesting it inhibits Chk1/2.

Incubation of MDA-MB-231 cells with SN38 caused phosphorylation of both Chk1 and Chk2 (Fig. 9). Upon addition of ICP-1, no change was observed in the phosphorylation status of either protein over the following 15 h, although the cells progressed through S, G_2, and eventually M during this time period. These results are similar to those obtained with UCN-01 (13) and demonstrate that ICP-1 does not act upstream of Chk1/2.

Cdc25C exists in three forms detectable on Western blots. The fastest migrating species is unphosphorylated (form "a" in Fig. 9), whereas a species with slightly retarded electrophoretic mobility represents phosphorylation at the inhibitory
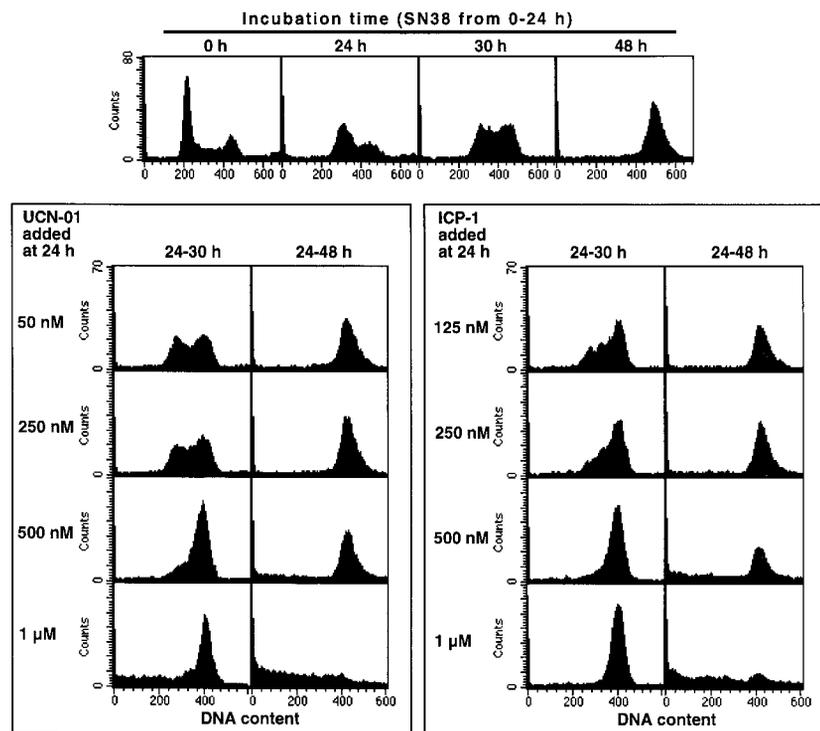
Fig. 8.   The influence of human plasma on ab-rogation of cell cycle arrest by UCN-01 and ICP-1. MDA-MB-231 cells were incubated with 10 ng/ml SN38 for 24 h, the drug was removed, and the cells were immediately harvested or in-cubated until harvest at 30 or 48 h (*top panel*). At 24 h, UCN-01 (*left panels*) or ICP-1 (*right panels*) was added at the indicated concentrations until harvest at 30 or 48 h. At harvest, cells were fixed and analyzed for cell cycle distribution by flow cytometry. All incubations between 24 and 48 h contained 5% human plasma plus 5% bovine serum.

serine 216 (form "b"); this is the predominant form in asyn-chronous cells. A third species with a significantly retarded mobility represents the hyperphosphorylated and active form of the protein (form "c"). We have also used two antibodies that recognize Cdk1/cyclin B phosphorylation sites in Cdc25C and that only detect antigen when a cell enters mitosis. An antibody to phosphothreonine 48-Cdc25C de-tects the hyperphosphorylated form of Cdc25C. The anti-body to phosphothreonine 67-Cdc25C detects several bands that migrate with an electrophoretic mobility similar to or slightly slower than the inactive form (13). The significance of these different forms is currently under investigation.

In asynchronously growing undamaged MDA-MB-231 cells, the majority of Cdc25C was in the phosphorylated, inactive form (Fig. 9). Incubation with 10 ng/ml SN38 for 24 h caused little change in these bands. Upon addition of 250 nM ICP-1, activation of Cdc25C was observed, but it is impor-tant to note that this did not occur until 9–12 h, which is the time that ICP-1 begins to drive the cells into mitosis. Hence, the abrogation of S-phase arrest that occurs 0–6 h after addition of 250 nM ICP-1 is not associated with activation of Cdc25C. However, at later times when the cells undergo mitosis, Cdc25C is activated as judged both by retarded mobility and by phosphorylation of threonines 48 and 67. These results are similar to those observed in cells incubated with UCN-01 (13), suggesting that the two analogues are likely to impact the same molecular target. Parallel experi-ments were performed in MCF10a cells: SN38 induced phosphorylation of Chk1 and Chk2, but did not activate Cdc25C, consistent with the failure of ICP-1 to abrogate cell cycle arrest in these cells (data not shown).
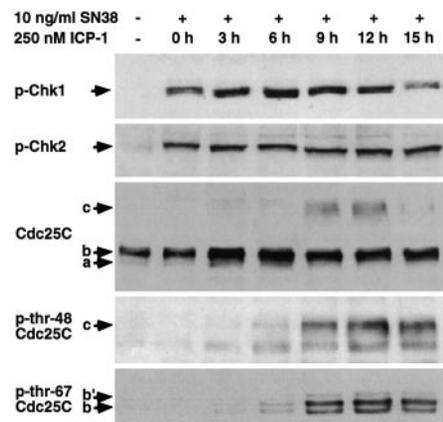

Fig. 9.   Analysis of cell cycle regulatory proteins Chk1, Chk2, and Cdc25C. MDA-MB-231 cells were incubated with 10 ng/ml SN38 for 24 h. The drug was removed, and the cells were incubated for an additional 3–15 h with 250 nM ICP-1. At each time point, cells were harvested, lysate was separated by gel electrophoresis, and Western blotting was performed with the indicated antibodies. The phosphorylation of Chk1 and Chk2 was detected using phospho-specific antibodies. The three forms of Cdc25C are unphosphoryl-ated inactive (*a*), phosphorylated inactive (*b*), and hyperphosphorylated ac-tive (*c*). The phosphothreonine 48 form of Cdc25C coelectrophoreses with form *c* while phosphothreonine 67 Cdc25C coelectrophoreses with form *b* or slightly slower (designated form *b'*).

Discussion

The use of drugs that overcome DNA damage-induced cell cycle arrest presents an exciting strategy for cancer chem-otherapy. UCN-01 was identified as a drug that could abro-gate cell cycle arrest and enhance cytotoxicity of several

DNA damaging agents (5, 6, 18) and recently entered clinical trials in various combination drug studies. Unfortunately, UCN-01 was found to bind avidly to human plasma proteins, possibly limiting its therapeutic potential (8, 9). UCN-01 is also not selective for checkpoint abrogation because it was initially discovered as a PKC inhibitor (20, 21). Accordingly, we began the search for alternate candidates that might have improved therapeutic potential. On the basis of our previous structure-activity studies discussed in the introduction, we synthesized a novel analogue of K252a that was modified at the 7-position (Fig. 1). We synthesized the 7-keto derivative initially as this has only a single isomer compared with the 7-hydroxylated version, which has four isomers. This 7-keto-K252a derivative has been called ICP-1.

In many of the experiments reported here, we find that ICP-1 acts similarly to UCN-01. Specifically, we find that both analogues abrogate S and G_2 arrest induced by either cisplatin or SN38 and enhance the cytotoxicity of these drugs in MDA-MB-231 cells. The efficacy for checkpoint abrogation differs: 250 nM ICP-1 appears to have equivalent efficacy to 10 nM UCN-01. In contrast, neither drug abrogated S or G_2 arrest in MCF10a cells. These results are consistent with previous reports that UCN-01 preferentially abrogates arrest in p53 mutant cells (6, 13, 18). We have extended these studies to analysis of four other breast cell lines, two with wild-type p53 (MCF7 and ZR75.1) and two mutant (T47D and MDA-MB-435), and find that only the p53 mutant cells undergo checkpoint abrogation (Ref. 10; data not shown).

The mechanism by which ICP-1 abrogates arrest also appears similar to UCN-01. Specifically, DNA damage-induced phosphorylation of Chk1 and Chk2, and neither UCN-01 nor ICP-1 caused their dephosphorylation, suggesting both drugs act further downstream. The ability of UCN-01 to abrogate cell cycle arrest is thought to be mediated by its inhibition of Chk1 kinase activity (22, 26). These reports suggested that Chk2 was not inhibited by UCN-01, but more recently, it has been shown that UCN-01 may be equally effective at inhibiting Chk2 (23). Upon incubation of S phase-arrested MDA-MB-231 cells with either UCN-01 or ICP-1, Cdc25C was not immediately activated; rather, the cells progressed through S phase and G_2 before they activated Cdc25C at mitosis. During S phase, Cdc25C is normally phosphorylated on serine 216 and inactive, and this phosphorylation is attributed to the constitutive activity of C-TAK1 (27). Hence, incubating S-phase cells with UCN-01 or ICP-1 may inhibit Chk1/2, but Cdc25C remains phosphorylated and inactive because of C-TAK1. Presumably, C-TAK1 is normally switched off or overwhelmed when a cell enters mitosis, and this would be the time when a damaged cell would rely on Chk1/2 to maintain Cdc25C in an inactive form. These results suggest the mechanism of S-phase abrogation by UCN-01 and ICP-1 may reflect the action of Chk1/2 on targets other than Cdc25C. However, the important conclusion for the present comparison is that the two drugs appear to act in a similar manner, suggesting they act on the same targets, presumably Chk1 and/or Chk2.

UCN-01 also has additional activities that are not apparent with ICP-1. We have previously reported that higher concentrations of UCN-01 (>200 nM) appear to inhibit C-TAK1 or another kinase that constitutively phosphorylates Cdc25C (13). The concurrent inhibition of Chk1/2 and C-TAK1 by UCN-01 can cause S phase-arrested cells to prematurely enter mitosis without progressing to G_2. We have looked at ICP-1 at concentrations up to 10 μM and have not observed this phenomenon, suggesting that it does not inhibit C-TAK1.

Another significant difference between UCN-01 and ICP-1 is observed when combined with araC. When UCN-01 was incubated concurrently with araC, it significantly enhanced cytotoxicity in both cell lines. In contrast, ICP-1 had no effect in combination with araC, demonstrating that this sensitization is not related to inhibition of Chks. As UCN-01 also inhibits PKC, we performed studies with two other PKC inhibitors, GF109203X and chelerythrine chloride, that do not abrogate cell cycle arrest (data not shown). Neither of these drugs enhanced the cytotoxicity of araC, suggesting that UCN-01 does not sensitize cells to araC through inhibition of PKC. Analysis of cell cycle perturbation in cells treated with this combination suggested the mechanism may relate to an ability of UCN-01 to increase araC activation or decrease its inactivation. The observation that UCN-01 enhanced araC-mediated cytotoxicity in both the cell lines used here is important because it suggests UCN-01 may sensitize both normal and tumor cells to araC.

One of the major limitations to the use of UCN-01 in patients is its avid binding to plasma proteins. The binding capacity of human plasma proteins at the MTD is 36 μM (9), yet only 10 nM is required to abrogate arrest and enhance cytotoxicity. Concentrations >100 nM are also cytotoxic to cells. It is therefore likely to be very difficult to administer just enough UCN-01 above plasma saturation to cause checkpoint abrogation without exceeding its cytotoxic concentration. To address this concern, we have performed experiments with human plasma in cell culture. In the case of UCN-01, enhanced cell killing was not observed in the presence of human plasma until 1 μM drug was added. Similar results were also observed with staurosporine (data not shown). Considering that we used 5% plasma in these experiments, this extrapolates to a binding capacity of 20 μM drug if whole plasma were used, which is within the range of plasma levels observed in patients receiving the MTD of UCN-01.

In contrast, we found that K252a abrogated S-phase arrest at 500 nM (10) and this was not prevented by human plasma (data not shown). This was part of the rationale for synthesizing ICP-1. Here we show that ICP-1 is only slightly inhibited by the presence of human plasma. Whereas in bovine serum, ICP-1 abrogated arrest at 250 nM in the presence of human plasma, and complete abrogation was seen at 1 μM. This raises an interesting point because UCN-01 and ICP-1 appear equieffective in human plasma. However, the partial abrogation of arrest seen with 250 nM ICP-1 in human plasma, which is the concentration that also abrogates arrest in bovine serum and in serum-free medium, suggests this binding to human serum is far weaker than for UCN-01. In addition, ICP-1 is less toxic to cells at these concentrations, which suggests that a greater excess over the concentration

that saturates plasma may be safely administered to patients.

Several recent publications have suggested that abrogation of DNA damage-induced checkpoints may not correlate with the p53 status of the cell (28, 29). There is also contradictory evidence as to whether the activity of these drug combinations requires abrogation of cell cycle arrest (28, 30). We have presented evidence here that concurrent UCN-01 can indeed enhance the cytotoxicity of araC in both p53 wild-type and mutant cells. However, sensitization was associated with earlier arrest in the cell cycle rather than later as would occur if a checkpoint was inhibited. Furthermore, this sensitization was not observed with ICP-1. Taken together, these results suggest that this sensitization to araC is unrelated to checkpoint abrogation. In other studies in which UCN-01 was added after SN38 or araC, we have observed enhanced cytotoxicity without the need for cell cycle progression, yet this only occurred at concentrations of UCN-01 above 200 nM (13). These concentrations are far above those needed to abrogate cell cycle arrest and suggest an alternate target such as C-TAK1 as discussed above. A number of other studies have used concentrations of UCN-01 between 0.1 and 1 μM (31–33), and the selective impact on checkpoint abrogation may be difficult to assess considering the inhibition of other targets at these concentrations. In contrast, ICP-1 did not appear to inhibit C-TAK1 as reflected in the fact that it did not drive S phase-arrested cells directly into mitosis. These studies emphasize the need to develop drugs with greater selectivity for the desired target, both to better understand the mechanisms of action, and to further develop this promising therapeutic strategy.

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Molecular Cancer Therapeutics

A Novel Indolocarbazole, ICP-1, Abrogates DNA Damage-induced Cell Cycle Arrest and Enhances Cytotoxicity: Similarities and Differences to the Cell Cycle Checkpoint Abrogator UCN-01

Alan Eastman, Ethan A. Kohn, Mary Kay Brown, et al.

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