ABSTRACT

The mechanisms of resistance to nucleoside analogues established in preclinical models are rarely found in primary tumors resistant to therapy with these agents. We tested the hypothesis that cells sense sublethal incorporation of analogues into DNA during replication and react by arresting further DNA synthesis and cell cycle progression. After removal of drug, cells may be able to repair damaged DNA and continue proliferation, thus escaping nucleoside analogue toxicity. As a corollary, we evaluated whether dysregulation of this mechanism causes cell death. Using gemcitabine as a model of S-phase-specific nucleoside analogues in human acute myelogenous leukemia ML1 cells, we found that DNA synthesis decreased, cells arrested in S-phase transit, and 60–70% of the population accumulated in S-phase in response to cytostatic conditions. Proliferation continued after washing the cells into drug-free medium. S-phase-arrested cells were then treated with otherwise nontoxic concentrations of UCN-01, which caused rapid onset of apoptosis without cell cycle progression specifically in cells with an S-phase DNA content. Thus, S-phase arrest by nucleoside analogues sensitizes cells to UCN-01, which appears to activate signaling for death mechanisms and/or inhibit survival pathways. These results differ from those in cells arrested at the G2 checkpoint, in which UCN-01 abrogates cell cycle arrest, permitting cells to progress in the cell cycle before apoptosis.

INTRODUCTION

DNA-directed nucleoside analogues, such as ara-C, fludarabine, and gemicitabine, are antimetabolites effective in the treatment of a variety of malignancies (1, 2). The major mechanism of action of nucleoside analogues is through incorporation into DNA, and thus this class of agents shows specificity for cells in S-phase (3–7). The triphosphates of nucleoside analogues compete with the cellular deoxyribonucleotides for incorporation into DNA during replication. Analogue incorporation inhibits DNA synthesis and kills the cells either by inducing apoptosis in cells capable of this process or by mitotic catastrophe in cells that lack the ability to conduct programmed cell death (8, 9). The precise mechanisms by which nucleoside analogues induce these forms of cell death are largely unknown.

Although ara-C has remained the mainstay of a variety of combination treatments for acute myelogenous leukemia (10–12), resistance to ara-C-based chemotherapy is still a major problem in therapy (13–15). Several in vitro-generated mechanisms of resistance to ara-C and other clinically active nucleoside analogues focus on their metabolism and cellular pharmacology (16–19). However, none appear prevalent in the leukemic cells of patients with resistant disease. Accordingly, no experimental models have been established for mechanisms of resistance that arise in response to nucleoside analogue incorporation into DNA. Because such incorporation appears essential to nucleoside analogue cytotoxicity (3–6), clinically relevant resistance mechanisms likely involve signaling processes that occur after the incorporation of the fraudulent nucleotides.

Most immortalized leukemic cells in S-phase die when exposed to cytotoxic concentrations of nucleoside analogues. However, when primary leukemic cells in the marrow are exposed to ara-C therapy, a substantial portion of them terminate DNA synthesis, arrest cell cycle progression, and accumulate in S-phase (20–26). Presumably after removal of the drug, cells resume progression and population growth. Thus, the ability of cells to enact a delay in cell cycle progression that stops DNA synthesis, thereby limiting incorporation of analogue, may be a defense mechanism that spares potential toxicity. These actions are similar to mechanisms that prevent cell cycle progression of cells in G1 and G2 so that damaged DNA can be repaired (27–29).

Several strategies have been devised to abrogate cell cycle arrest caused by therapeutic agents (30–34). For example, 2-aminopurine may override multiple cell cycle checkpoints (30, 31). In addition, caffeine has been used to preferentially abrogate G2 arrest and enhance cytotoxicity of DNA-damaging anticancer agents, such as cisplatin and ionizing radiation (35–38). Unfortunately, the caffeine concentration required to achieve this effect in cell culture (5 mm) exceeds clinically tolerable levels by >50-fold (39). On the other hand, the kinase inhibitor UCN-01 can abrogate the G2 checkpoint induced by cisplatin or ionizing radiation and promote cell cycle progression to mitosis at 25–100 nm (32, 40), a concentration of free drug that can be achieved in clinical studies (41, 42). Although originally developed as an inhibitor of protein kinase C (43, 44), UCN-01 appears to target Chk1 in G2-arrested cells. Chk1 is a cell cycle checkpoint kinase that indirectly affects the activity of Cdc2/cyclin B, which is required for exit from G2 into mitosis (45, 46). Although less understood, UCN-01 may also enhance cytotoxicity and abrogate a combined S-phase and G2 phase arrest induced by campothecin in HT29 human colon carcinoma cells (47), by cisplatin in Chinese hamster ovary cells (48), and by mitomycin C in A431 epidermoid carcinoma cells and MCF-7 breast carcinoma cells (49).

On the basis of these observations, we hypothesized that nucleoside analogues arrest cell cycle progression of the S-phase population to block further incorporation of analogue molecules into DNA. In the absence of nucleoside analogue, the nucleotides are cleared from the cell, and DNA damaged by incorporation is repaired. Thereafter, surviving cells are capable of proliferation. Thus, the ability of cells to enact an S-phase arrest in response to incorporation of nonlethal amounts of nucleoside analogue may serve as a mechanism of resistance to S-phase-specific agents. As a corollary, the dysregulation of S-phase arrest may contribute to analogue cytotoxicity. Here we characterize an S-phase arrest induced by the pyrimidine nucleoside analogue gemicitabine in a human myelogenous leukemia cell line and report the actions of UCN-01 on cell cycle progression and viability of this population.
MATERIALS AND METHODS

Cell Culture. ML-1, a human acute myelogenous leukemia cell line containing wild-type p53, was a gift from Dr. Michael B. Kastan (St. Jude Children’s Research Hospital, Memphis, TN). CCRM-CEM, a human T-lymphoblastic leukemia cell line containing two mutated p53 alleles, was obtained from the American Type Culture Collection (Rockville, MD). Cell lines were maintained in exponential growth phase in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc., Grand Island, NY) for ML-1 cells and 5% serum for CCRM-CEM cells. Cells were incubated at 37°C (5% CO2) in a humidified atmosphere. Population-doubling times were approximately 22–24 h.

Chemicals and Antibodies. The nucleoside analogue gemcitabine was kindly provided by Dr. L. W. Hertel (Lilly Research Laboratories, Indianapolis, IN). ara-C was purchased from Sigma Chemical Co. (St. Louis, MO). F-ara-A was produced by alkaline phosphatase treatment of fludarabine, which was obtained from Berlex Laboratories (Richmond, CA). FMdC was from Hoechst Marion Roussel (Bridgewater, NJ). UCN-01 (NSC 638850) was kindly provided by the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). Aliquots of UCN-01 (10 mM in DMSO) were stored at −20°C and diluted in water immediately prior to each experiment. All other chemicals were reagent-grade.

[2-3H]Thymidine (50 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). [ methyl-3H]Thymidine (5 Ci/mmol) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). [γ-32P]ATP (4500 Ci/ mmol) was purchased from ICN Radiochemicals (Costa Mesa, CA).

Goat polyclonal antibody to Bcl-XL (sc-7122) and rabbit polyclonal antibody to Bax (sc-493) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to Bcl-2 (1624 989) was purchased from PharMingen International (San Diego, CA). Mouse monoclonal antibody to Bax (sc-493) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal antibody to Bcl-2 (1624 989) was purchased from PharMingen International (San Diego, CA). Mouse monoclonal antibody to Bcl-XL (sc-7122) and rabbit polyclonal antibody to caspase-3 were purchased from PharMingen International (San Diego, CA). Mouse monoclonal antibody to β-actin was purchased from Sigma. Antiimmune IgG horseradish peroxidase-conjugated antibody was obtained from Bio-Rad Laboratories (Hercules, CA). Antirabbit IgG horseradish peroxidase-conjugated antibody was obtained from Oncogene Research Products (Cambridge, MA).

DNA Content Analysis by PI Staining. Flow cytometric analysis of PI-stained cells was performed to demonstrate the effects of gemcitabine and UCN-01 on cell cycle progression. Briefly, cells were harvested at each time point, washed twice with ice-cold PBS, and fixed in 70% ethanol at least overnight at 4°C. Before flow cytometry, cells were washed twice with ice-cold PBS and stained with 1 ml of PI (15 μg/ml; Sigma) containing 2.5 μg/ml RNase A (Boehringer Mannheim) for at least 30 min. DNA content of at least 20,000 cells was determined by FACScan Coulter Epics XL-MCL flow cytometer (Coulter Corp., Hialeah, FL). The proportion of cells in a particular phase of the cell cycle was determined by a Multicycle program (Coulter).

Cell Cycle Analysis by BrdUrd/PI Staining. ML-1 cells were pulse-treated with 10 μM BrdUrd (Sigma) for 30 min at 37°C and washed twice with 1% BSA/PBS to remove unincorporated BrdUrd. Cells were then replenished with fresh, prewarmed medium and either treated with gemcitabine or left untreated as a control. At the indicated time points, cells were washed twice with 1% BSA/PBS and fixed with 70% cold ethanol for at least 24 h. Double helical DNA was acid denatured with 2 N HCl/0.5% Triton X-100 for 30 min at room temperature. It was then neutralized with 0.1 M Na2B4O7 (pH 8.5) for 15 min. Cells were then washed with 0.5% Tween 20/1% BSA/PBS and resuspended in 50 μl of the same solution. They were then incubated with 20 μl of FITC-conjugated monoclonal anti-BrdUrd antibody (Becton Dickinson, San Jose, CA) per 1 × 106 cells for 30 min at room temperature on a rocker. Cells were washed with 0.5% Tween 20/1% BSA/PBS and stained with 1 ml of PI (15 μg/ml) containing 2.5 μg/ml RNase A for at least 30 min.

Examination of Cell Morphology. After centrifugation of 5 × 106 cells to a slide by cytofix (550 rpm for 5 min), cells were fixed with 100% methanol for 20 min, air-dried, and stained by Wright’s Giemsa stain solution (Fisher Scientific, Pittsburgh, PA) for 15 min. Cell morphology was examined by light microscopy using a Nikon HFX-II microscope. Apoptotic morphology was identified by the appearance of cell shrinkage, nuclear condensation, and/or the appearance of membrane-bound apoptotic bodies. At least 500 cells/field in a minimum of three randomly selected fields were counted on three slides for each sample.

Quantification of DNA Fragmentation. DNA fragmentation was quantified as described previously (50) with modifications (51). Briefly, exponentially growing ML-1 cells were prelabeled with [3H]thymidine for 24 h (0.01 μCi/ml every 12 h) at 37°C and treated with gemcitabine ± UCN-01. At harvest, 2–4 × 106 cells were pelleted, washed with Ca2+/Mg2+-free PBS, and lysed by incubation in 1 ml of hypotonic lysis buffer containing 10 mM Tris-Cl (pH 7.5), 1 mM EDTA, and 0.2% Triton X-100 (Sigma) on ice for 20 min. Intact chromatin-sized DNA (insoluble and in the pellet) was separated from fragmented DNA (soluble at low-salt concentrations and in the supernatant) by centrifugation at 13,000 × g for 10 min. The pelleted chromatin was solubilized in 1 ml of Soluene-350 (Packard Instrument Company, Meriden, CT) at 60°C for 2 h. The radioactivities of both the pellet and supernatant fractions were determined in 10 ml of Liquiscint scintillation fluid (Packard) by a liquid scintillation counter (Packard). The percentage of DNA fragmentation was calculated as:

\[
\text{% DNA fragmentation} = \frac{\text{Radioactivity (dpm) of supernatant} \times 100}{\text{Radioactivity (dpm) of supernatant and pellet}}
\]

Gel electrophoresis was used to visually identify DNA that had been enzymatically digested into the ladder pattern of oligonucleosome-sized DNA fragments.

DNA Synthesis Assay. Exponentially growing ML-1 cells were prelabeled with [3H]thymidine for 24 h (0.01 μCi/ml every 12 h) at 37°C and incubated with 10 mM gemcitabine ± 100 mM UCN-01 for the indicated times in a 96-well filtration plate (Millipore Corp., Bedford, MA). [ methyl-3H]Thymidine was added (0.4 μCi/well) at least 30 min before incubation ended, and labeled cells were collected on filters using a vacuum manifold (Millipore). The filters were washed four times each with 8% trichloroacetic acid, Millipore water, and 100% ethanol. They were then dried and punched into a vial. Radioactivity retained on the filter was determined by a liquid scintillation counter (Packard). H values were normalized by the [3H]radioactivity.

TUNEL Assay. To identify the cell cycle position of apoptotic cells, a flow cytometric TUNEL assay was performed using an APO-DIRECT kit (Phoenix Flow Systems, Inc., San Diego, CA) according to the manufacturer’s protocol. Each sample was analyzed on a FACSCan Coulter Epics XL-MCL flow cytometer (Coulter); red PI fluorescence was acquired on a linear scale on the X axis, and green FITC staining was acquired on a log scale on the Y axis.

Cell Lysis. Briefly, 1–3 × 106 cells were pelleted by centrifugation at 1500 rpm for 5 min, washed twice with ice-cold PBS, and lysed on ice for 20 min in lysis buffer containing 25 mM HEPEs (pH 7.5), 300 mM NaCl, 1.5 mM MgCl2, 0.5 mM sodium deoxycholate, 20 mM β-glycerophosphate, 1% Triton X-100, 0.1% SDS, 0.2 mM EDTA (pH 8.0), 0.5 mM DTT, 1 mM sodium orthovanadate (pH 10), 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin. Cells were centrifuged at 14,000 rpm at 4°C for 15 min, and the supernatant was stored at −70°C until use. Protein content was determined using a protein assay kit according to the manufacturer’s instructions (Bio-Rad Laboratories).

Immunoblotting Analysis. An equal volume of 2× SDS sample-loading buffer containing 100 mM Tris-Cl (pH 6.8), 20% glycerol, 4% SDS, 0.05% bromophenol blue, and 5% β-mercaptoethanol was added to cell lysates. Lysates were then heated at 95–100°C for 5 min. Ninety-mg aliquots of total cellular protein were loaded onto 8–12% SDS-polyacrylamide gels (percentages depended on protein sizes detected), and proteins were electrophoresed at constant voltage (70–100 V) and electrotransferred to Immobilon-P membranes (Millipore) for 1–3 h at 250 mA. Membranes were blocked overnight in TBS-Tween 20 containing 5% nonfat dried milk and then incubated with primary antibodies (0.5–2 μg/ml) for 3 h, and secondary antibodies were conjugated to horseradish peroxidase (1:2500 dilution) for 1 h. The blots were visualized by enhanced chemiluminescence according to the manufacturer’s instructions (Pierce, Rockford, IL).

Immunoprecipitation and Histone H1 Kinase Assay. For each sample, 500 μg of cell protein were mixed with 3 μg of rabbit polyclonal Cdc2 antibody on a rocker for 3 h at 4°C. The immune complexes were then mixed with 100 μl of 20% protein A-Sepharose beads (Amersham Pharmacia Biotech) on a rocker for another 1–2 h at 4°C, and the beads were washed twice with lysis buffer and once in kinase buffer containing 20 mM Tris-Cl (pH 7.5), 0.1 mM EGTA (pH 7.0), 10 mM MgCl2, and 1 mM DTT. The Cdc2 immunoprecipitates were incubated with 30 μl of kinase buffer plus 20 μM cold...
ATP, 3 μg of histone H1 (Boehringer Mannheim), and 6 μCi of [γ-32P]ATP for 30 min at 37°C. An equal volume of 2× SDS sample-loading buffer was added to terminate the reaction. The mixture was then boiled for 5 min and loaded onto a 10% SDS-polyacrylamide gel. Autoradiography was performed, and radioactivities were quantified by a Betascope 603 blot analyzer (Betagen, Waltham, MA).

RESULTS

S-Phase Arrest Induced by Cytostatic Concentrations of Nucleoside Analogues in Exponentially Growing ML-1 Cells. To develop a model for S-phase arrest in exponentially growing ML-1 cells, we first investigated the effects of cytostatic concentrations of nucleoside analogues on the cell cycle. Fig. 1A shows the typical cell cycle distribution of an exponentially growing population of ML-1 cells; the S-phase fraction is ~33%. In contrast, incubation of cells for 24 h (approximately one population doubling time) with either 10 nM gemcitabine, 5 nM FMdC, 50 nM ara-C, or 2 μM F-ara-A resulted in an increase in the S-phase fraction to 60–70% (Fig. 1, B–E, respectively). Treatments were minimally toxic to the cells, because there was a <10% increase in sub-G1 populations. Thus, the concentrations of nucleoside analogues used were cytostatic and caused an accumulation of cells in S-phase. Hereafter, although our data suggested that the cellular response was similar for each of these nucleoside analogues tested, we focused our study on gemcitabine.

To further characterize gemcitabine-induced S-phase arrest, we first determined the dose response for S-phase accumulation in exponentially growing populations (Fig. 2A). Maximum S-phase arrest for a 24-h gemcitabine treatment occurred with 6–15 nM gemcitabine. Lower concentrations failed to arrest cells, whereas higher concentrations caused accumulation at the G1–S boundary and most likely killed S-phase cells. Thus, we chose 10 nM gemcitabine to induce S-phase arrest in ML-1 cells for additional experiments. S-phase accumulation reached a plateau after 16 h of incubation and was maintained through 34 h (Fig. 2B). To evaluate the stability of this arrest, cells were pulsed with BrdUrd for 30 min, washed into fresh medium, and incubated with 10 nM gemcitabine. For cells not treated with gemcitabine, those in S-phase at the time of BrdUrd labeling exited S-phase by 9 h and cycled back to the subsequent S-phase by 18 h. In contrast, during continuous treatment with gemcitabine for as long as 34 h, <25% of...
S-phase cells were able to transit out of S-phase (Fig. 2C). The significant delay in progression through S-phase during treatment with a cytostatic concentration of gemcitabine was consistent with an effective cell cycle arrest.

Next, we determined whether S-phase-arrested cells could proliferate after removal of gemcitabine, as would be expected in a resistant phenotype. Growth was quantitated after cells were treated with 10 nM gemcitabine for 24, 36, and 48 h and washed into fresh medium. Incubation for 24–36 h caused a major arrest of the population in S-phase. However, after the analogue was washed out, cells continued proliferating at a rate comparable with controls, although proliferation restarted after lag times that correlated with the times spent in gemcitabine. Cells treated with gemcitabine for 48 h (more than two population doubling times) did not recover proliferative capacity (Fig. 3A). The absence of BrdUrd-positive cells with a sub-G1 DNA content (data not shown) and the maintenance of cell numbers (detected by particle counts) suggested that the treatment did not cause cell lysis.

Lack of Cell Cycle Progression after UCN-01 Treatment of Cells Arrested in S-Phase by Gemcitabine. Prior studies have indicated that UCN-01 can abrogate cell cycle blockage of cells arrested in G2 by ionizing radiation (40), cisplatin (32), or DNA topoisomerase I inhibitors (47). In this study, we investigated the actions of UCN-01 on cells arrested exclusively in S-phase. Dose-response experiments demonstrated that continuous incubation with 500 nM UCN-01 had no effect on the viability of ML-1 cells after 72 h; therefore, we chose 100 nM UCN-01 for further investigation, because it was clearly nontoxic and had no effect on cell cycle progression (data not shown). Addition of 100 nM UCN-01 to ML-1 cells after a 24-h incubation with 10 nM gemcitabine resulted in a rapid decrease in the S-phase fraction (Fig. 3A). If this promoted cell cycle progression, we would expect the G2 fraction to begin to expand as the S-phase population diminished. However, no increase in the G2 population was evident after UCN-01 addition (Fig. 3B), and the G1 population appeared stable (Fig. 3C). Rather, there was a concomitant increase in cells with a sub-G1 DNA content (Fig. 3D). These results suggested that addition of an otherwise nontoxic concentration of UCN-01 to S-phase-arrested cells initiated cell death without cell cycle progression.

If UCN-01 were to promote cell cycle progression of the S-phase-arrested cells induced by cytostatic treatment with gemcitabine, it would be expected that cells would reinitiate DNA synthesis. To evaluate this possibility, we quantitated DNA synthesis in gemcitabine-arrested cells before and after UCN-01 addition (Fig. 4A). Gemcitabine treatment for 24 h inhibited DNA synthesis by 65%, and synthesis did not increase after addition of UCN-01. In fact, there was a small but reproducible decrease to only 7% of control values, indicating that DNA synthesis was not reactivated by UCN-01. In addition, immunoprecipitated Cdc2 kinase did not increase in gemcitabine-arrested cells or after addition of UCN-01 (Fig. 4B). Because activated Cdc2 kinase is essential for cell cycle progression through G2 into mitosis, these results supported the conclusion that UCN-01 did not promote the ability of S-phase-arrested cells to pass through the cell cycle. Finally, there was no increase in the absolute number of cells in the culture within 10 h after UCN-01 addition (data not shown).

Cells arrested in S-phase by F-ara-A or ara-C showed similar responses to UCN-01, suggesting a general nature of this response (data not shown). In parallel studies, human T-lymphoblastic leukemia CCRF-CEM cells, which contain two mutated p53 alleles, accumulated in S-phase in response to cytostatic treatments with gemcitabine and F-ara-A. Subsequently, UCN-01 also specifically decreased the S-phase population in these nucleoside analogue-arrested cells (data not shown). This suggested that the ability of nucleoside analogues to arrest cells in S-phase and the effects of UCN-01 on these populations were both independent of p53 status.

**Induction of Apoptosis by UCN-01 in Gemcitabine-arrested ML-1 Cells.** The marked increase in the sub-G1 population of S-phase-arrested cells after a 10-h incubation with 100 nM UCN-01 (Fig. 4A) suggests that this drug could promote cell death. Percentage of viable cells in the sub-G1 population was increased 6.5-fold at 10 nM UCN-01 and 11-fold at 100 nM UCN-01 (Fig. 4A). The marked increase in the sub-G1 population of S-phase-arrested cells (data not shown) indicated that induction of apoptosis by UCN-01 was rapid and occurred before the cells were washed out of the sub-G1 DNA content region by flow cytometry.

**Growth in the absence of gemcitabine.** Cells incubated with 10 nM gemcitabine resulted in a rapid decrease in the S-phase population (Fig. 3A). The absence of BrdUrd-positive cells with a sub-G1 DNA content (data not shown) and the maintenance of cell numbers (detected by particle counts) suggested that the treatment did not cause cell lysis.

**Lack of Cell Cycle Progression after UCN-01 Treatment of Cells Arrested in S-Phase by Gemcitabine.** Prior studies have indicated that UCN-01 can abrogate cell cycle blockage of cells arrested in G2 by ionizing radiation (40), cisplatin (32), or DNA topoisomerase I inhibitors (47). In this study, we investigated the actions of UCN-01 on cells arrested exclusively in S-phase. Dose-response experiments demonstrated that continuous incubation with 500 nM UCN-01 had no effect on the viability of ML-1 cells after 72 h; therefore, we chose 100 nM UCN-01 for further investigation, because it was clearly nontoxic and had no effect on cell cycle progression (data not shown). Addition of 100 nM UCN-01 to ML-1 cells after a 24-h incubation with 10 nM gemcitabine resulted in a rapid decrease in the S-phase fraction (Fig. 3A). If this promoted cell cycle progression, we would expect the G2 fraction to begin to expand as the S-phase population diminished. However, no increase in the G2 population was evident after UCN-01 addition (Fig. 3B), and the G1 population appeared stable (Fig. 3C). Rather, there was a concomitant increase in cells with a sub-G1 DNA content (Fig. 3D). These results suggested that addition of an otherwise nontoxic concentration of UCN-01 to S-phase-arrested cells initiated cell death without cell cycle progression.

**ACTIONS OF UCN-01 ON S-PHASE-ARRESTED CELLS**

**Fig. 3. Effects of UCN-01 on gemcitabine-arrested ML-1 cells.** Cells were incubated with 10 nM gemcitabine continuously for 34 h (●), and 100 nM UCN-01 was added at 24 h to one portion (○) when the culture was split. Cells were harvested at indicated times for analysis by flow cytometry. Quantitative data of relative DNA content from at least three independent experiments are shown: A, cells in S-phase; B, cells in G2-M phase; C, cells in G1 phase; and D, cells with a sub-G1 DNA content. Bars, SD.
3D) suggested that UCN-01 induced cell death. We therefore studied the cellular morphology of these cells and found cell membrane blebbing, nuclear condensation, and apoptotic bodies consistent with apoptosis (data not shown). Apoptotic cells were counted every 2 h after addition of 100 nM UCN-01, and the proportion of cells with apoptotic morphology increased over time during combination treatment with gemcitabine and UCN-01, rising to ~50% after 10 h (Fig. 5A). As an additional parameter of apoptosis, nucleosomal DNA fragmentation assays showed that DNA fragments appeared in gemcitabine-arrested cells 2 h after UCN-01 addition and increased with time (data not shown). To quantitate the DNA fragmentation, we labeled ML-1 cells with [14C]thymidine prior to gemcitabine treatment (data not shown). To quantitate the DNA fragmentation, we labeled ML-1 cells with [14C]thymidine prior to gemcitabine treatment and determined the percentage of soluble DNA fragments relative to the whole DNA content of the cultures (Fig. 5B). After addition of UCN-01, the induction of DNA fragmentation occurred at approximately the same time as the appearance of apoptotic morphology.

Consistent with apoptosis, immunoblotting demonstrated activation of the executioner caspase, caspase-3, after addition of UCN-01 to gemcitabine-treated ML-1 cells, as illustrated by the cleavage of caspase-3 from its M₆ 32,000 proenzyme form to a M₆ 17,000 fragment (Fig. 5C). This was associated with the cleavage of the caspase-3 substrate, PARP, into an M₆ 85,000 fragment. These results suggested that UCN-01 initiated an apoptotic cascade in the S-phase-arrested cells. Finally, because Bcl-2 family members may play a critical role in regulating cell survival or death, we examined whether the protein expression of some of these family members was affected by cytotoxic treatment with gemcitabine or by addition of UCN-01. Immunoblotting analysis of Bcl-2 and Bcl-Xₐ, which are inhibitors of cell death, and of Bax, which is a promoter of cell death, showed that protein levels were not affected by incubation with cytostatic concentration of gemcitabine or addition of UCN-01 (Fig. 5D).

Selective Killing of S-Phase-arrested Cells by UCN-01. The rapid loss of cells with an S-phase DNA content (Fig. 3A), the absence of an increase in the G₂ population (Fig. 3B), and the concomitant increase in cells with apoptotic markers after addition of UCN-01 (Fig. 3D) suggested that UCN-01 selectively killed the S-phase population. To evaluate this possibility, we used the TUNEL assay. Only a minor fraction of the population appeared to be TUNEL positive after 24-h cytostatic exposure to gemcitabine (Fig. 6A). However, a time course investigation after UCN-01 addition demonstrated that the TUNEL-positive cells appeared to arise from the portion of the population defined by PI staining to have an S-phase DNA content. Quantitation of these data indicated that the percentage of TUNEL-positive cells increased from 10% to >50% in the 10 h after UCN-01 addition (Fig. 6B). In contrast, the number of TUNEL-positive cells did not significantly increase with continued incubation with gemcitabine alone for as long as 34 h. Finally, the TUNEL-negative populations in the cells with an S-phase DNA content reciprocally decreased over time after the addition of UCN-01 (Fig. 6C). Because there was no significant change in TUNEL-negative cells in G₁ and G₂ (data not shown), we concluded that UCN-01 induced a rapid onset of apoptosis specific for the cells arrested in S-phase.

DISCUSSION

In the present study, we demonstrated that cytostatic concentrations of nucleoside analogues arrested cell cycle progression in S-phase and decreased DNA synthesis in human myeloblastic ML-1 cells and that removal of the analogues permitted renewed proliferation. However, although nontoxic concentrations of UCN-01 alone had no effect on population growth or cell cycle distribution of ML-1 cells, UCN-01 abruptly abrogated the survival of S-phase-arrested cells, thereby contributing to overcoming potential resistance to nucleoside analogues. Furthermore, this abrogation was not associated with cell cycle progression to the G₂-M phase. Rather, UCN-01 appeared to directly initiate an apoptotic cascade in S-phase-arrested cells.

Cell cycle checkpoints serve as surveillance systems to interrupt cell cycle progression when damage to the genome or spindle is detected or when cells have failed to complete a preceding event (52). Thus far, emerging studies have emphasized DNA damage-induced G₁ or G₂ checkpoints. However, little is known about the molecular mediators of S-phase-arresting mechanisms in mammalian cells. Although previous investigators have used agents that are not entirely specific for S-phase cells, such as cisplatin and camptothecin, we chose to use the nucleoside analogue gemcitabine because nucleoside analogues act by incorporating into DNA during replication, and therefore more specifically target cells in S-phase.

Although cytotoxic concentrations of gemcitabine and other nucleoside analogues selectively kill S-phase cells in human leukemia cell lines (8, 9), substantial literature demonstrates that not all S-phase cells are killed during cytotoxic chemotherapy. One goal of early therapeutic studies was to use the S-phase-specific nucleoside ara-C to synchronize the population of leukemic blasts in S-phase. Flow cytometry has clearly demonstrated an accumulation of cells in S-phase, indicating the resistance of at least a portion of the S-phase population to potentially lethal cell cycle-specific treatment (20–26). The high incidence of leukemic regrowth, even after intensive ara-C therapy that should have extended beyond the cell cycle time of most human leukemias (53, 54), suggests a mechanism of clinical resistance that is
not seen in metabolic models (55, 56). In the clinic, it is possible that blasts fully in S-phase could encounter low-level incorporation of the analogue into DNA at the beginning of infusions, when triphosphate concentrations are low. Additionally, some cells may transit into S-phase some time after the end of nucleoside infusion, when metabolic elimination has reduced the cellular level of triphosphate analogues. Here, we have used cytostatic treatment of an acute myelogenous leukemia cell line as a model of arrest during S-phase progression to evaluate it as a defense response to nonlethal analogue incorporation.

We found relatively narrow ranges of concentrations of each nucleoside analogue that optimized S-phase accumulation in the absence of cytotoxicity (Fig. 1 and data not shown), as illustrated specifically by gemcitabine (Fig. 2A). Presumably, the differences in concentrations among the analogues reflect their cellular pharmacology, leading to incorporation of a critical amount of each analogue in DNA, and possibly indicate differences in affinity of the putative sensor for a specific nucleotide. Because nucleotides of gemcitabine, fludarabine, and FMdC inhibit ribonucleotide reductase, it is possible that an imbalance in deoxynucleotide pools may also contribute to the S-phase arrest of cells incubated with these nucleosides. After release from S-phase arrest, populations exhibited a lag that reflected the duration of nucleoside analogue exposure before resuming proliferation at a rate comparable with controls (Fig. 2D). This suggested that an S-phase checkpoint had been activated, in which cell cycle progression through the S-phase at its normal rate was prevented, and incorporation of additional analogues was subsequently blocked. It is likely that the lag prior to continued proliferation reflects the time needed for elimination of cytostatic concentrations of the analogue nucleotides and repair of damage associated with incorporated drug and stalled replication forks. Thus, the induction of cytostasis and subsequent recovery may model a mechanism of resistance to nucleoside analogues. Furthermore, activation of the S-phase checkpoint by gemcitabine appeared independent of p53 status, because S-phase arrest was also observed in the p53 mutant cell line CCRF-CEM (data not shown).

The progression of cells through the cell cycle is controlled by core cell cycle regulators, the most prominent of which are a family of kinases under the direction of their regulatory subunits, the cyclins. Although these cyclin-dependent kinases have specific substrates that enable orderly progression through the cell cycle, a complex hierarchy of other kinases and phosphatases provides an overlay of regulation on these processes. In particular, the activity of the S-phase-specific complex of cyclin-dependent kinase 2 (Cdks2) and cyclin A is enabled by phosphorylation of Thr-160 and inhibited by phosphorylation of Thr-14 and Tyr-15 on Cdks2. The Thr-15 phosphorylation is antagonistically regulated by wee1 kinase and Cdc25A/Cdc25B phosphatases (57–59), the direct upstream regulators of Cdks2. Recent studies have shown that the checkpoint kinases Chk1 and Chk2 are upstream serine/threonine protein kinases that regulate both wee1 kinase and Cdc25A/B phosphatase activity (60, 61). Chk2 becomes phosphorylated and activated in response to DNA damage or replication blockage...
were no cells coming from the S-phase and that cells previously in G2 did not increase but rather decreased, indicating that population of cells with S-phase DNA content decreased, the fraction instead decreased after the addition of UCN-01 (Fig. 4).

The Cdc25C pathway as potential targets of G2 checkpoint abrogation studies from two different groups both identified the Chk1 kinase and replication checkpoint in the absence of Chk2 (67, 68). More recently, checkpoint (61, 65, 66) and serves an essential function in the DNA replication pathway of the Cdc25C pathway, perhaps functioning through Chk1/Chk2 and the Cdc25A/B pathway, may be involved in S-phase arrest induced by nucleoside analogues and dysregulated by UCN-01 addition.

Recently, several laboratories have reported that UCN-01 potently abrogates the G2 checkpoint (32, 40) and possibly the S-phase checkpoint (47–49) by allowing arrested cells to progress into the next phase of the cell cycle. However, for ML-1 cells arrested in S-phase by nucleoside analogues, we demonstrated that UCN-01 addition affected the S-phase-arrested fraction specifically, and that these cells underwent apoptosis without cell cycle progression: (a) DNA synthesis determined by [3H]thymidine incorporation was not increased but instead decreased after the addition of UCN-01 (Fig. 4A); (b) as the population of cells with S-phase DNA content decreased, the fraction of G2 cells did not increase but rather decreased, indicating that there were no cells coming from the S-phase and that cells previously in G2 may have moved to the G1 phase (Fig. 3B); (c) Cdc2 kinase activity did not increase after the addition of UCN-01 to gemcitabine-arrested cells, suggesting that cells were not transiting from G2 to mitosis (Fig. 4B); and (d) the TUNEL flow cytometric assay, used to determine the cell cycle specificity of apoptotic cells, demonstrated that it was the S-phase fraction that was selectively induced to undergo apoptosis (Fig. 6).

The molecular mechanisms for UCN-01-induced apoptosis in the S-phase-arrested cells are not fully understood. It has been shown that in F-ara-A-arrested cells, signaling through c-Jun NH2-terminal kinase is activated shortly after UCN-01 addition but before the cells begin to show biochemical or morphological evidence of apoptosis (70). Whether this pathway is also activated by gemcitabine needs further investigation. On the other hand, it has also been suggested that suppression of survival signaling pathways is involved in this treatment system. Recent evidence has shown that activation of the phosphatidylinositol 3-kinase-Akt kinase signaling pathway culminates in the phosphorylation of the Bcl-2 family member BAD, thereby suppressing apoptosis and promoting cell survival (71–73). Our preliminary results indicated that UCN-01-mediated changes in the phosphorylation status inhibited the phosphatidylinositol 3-kinase-Akt-BAD survival pathway and were associated with induction of apoptosis in gemcitabine-arrested cells (74).

In conclusion, the lack of metabolic resistance characteristics established in cell lines and in the leukemia blasts isolated from patients refractory to nucleoside analogue therapy prompted our hypothesis that S-phase arrest occurs in response to minimal damage. In our model, we demonstrate that ML-1 cells arrest cell cycle progression in S-phase in response to a variety of S-phase-specific analogues but are able to continue proliferation after removal of the drugs. Incubation with otherwise nontoxic concentrations of UCN-01 causes a rapid onset of apoptosis without cell cycle progression in nucleoside analogue-arrested cells. Thus, S-phase arrest appears to sensitize cells to actions of UCN-01 that inhibit survival pathways and/or activate cell death mechanisms. Based in part on these findings, a clinical trial of ara-C in combination with UCN-01 has been initiated in patients with relapsed acute myelogenous leukemia.

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REFERENCES


S-Phase Arrest by Nucleoside Analogues and Abrogation of Survival without Cell Cycle Progression by 7-Hydroxystaurosporine
