DNA damage responses triggered by a highly cytotoxic monofunctional DNA alkylator, hedamycin, a pluramycin antitumor antibiotic

Lan Chun Tu,[1] Thomas Melendy,[2] and Terry A. Beerman[1]

[1]Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Buffalo, New York and [2]Witebsky Center for Microbial Pathogenesis and Immunology and Departments of Microbiology and Biochemistry, University at Buffalo School of Medicine and Biomedical Sciences, Buffalo, New York

Abstract

Long-term exposure (72 h) to hedamycin, a monofunctional DNA alkylator of the pluramycin class of antitumor antibiotics, decreased growth of mammalian cells by 50% at subnanomolar concentrations. Short-term treatment (4 h) rapidly reduced DNA synthesis by 50% also at subnanomolar concentrations, but substantially higher levels were needed to block RNA synthesis while protein synthesis even at very high hedamycin concentrations remained unaffected. Hedamycin treatment at concentrations below its growth IC_{50} induced only a transient and temporary accumulation of cells in G_2. Somewhat higher concentrations resulted in substantial S-phase arrest, and at increasing concentrations, complete cell cycle arrest in G_1 was observed without the appearance of a sub-G_1 cell population. Neither inhibition of cell growth nor cell cycle arrest appeared to be dependent on ataxia and Rad-related kinase expression. DNA damage checkpoint proteins including p53, chk1, and chk2 were differentially activated by hedamycin depending on the concentration and duration of treatment. The level of downstream cell cycle regulators such as cdc25A, E2F1, cyclin E, and p21 were also altered under conditions that induced cell cycle arrest, but atypically, p21 overexpression was observed only in S-phase-arrested cells. Apoptotic indicators were only observed at moderate hedamycin concentrations associated with S-phase arrest, while increasing concentrations, when cells were arrested in G_1, resulted in a reduction of these signals. Taken together, the responses of cells to hedamycin are distinct with regard to its effect on cell cycle but also in the unusual concentration-dependent manner of activation of DNA damage and cell cycle checkpoint proteins as well as the induction of apoptotic-associated events. [Mol Cancer Ther 2004;3(5):577–85]

Introduction

When cells encounter DNA damage, a cascade of signal transduction pathways activate cell cycle checkpoints leading to blocked cell cycle progression, inhibition of DNA replication, and recruitment of repair proteins to the damaged DNA site (1, 2). Typically, damage in the form of DNA double-strand breaks induced by ionizing radiation is mediated by the phosphatidylinositol 3-kinase-related kinase ataxia telangiectasia mutated, which passes signals to downstream protein kinases leading to activation of cell cycle checkpoints (3). Information regarding responses to DNA alkylators that stall DNA replication is more limited partly due to the variety of agents, which stall replication forks including compounds that block chain elongation (*e.g.*, hydroxyurea), modify DNA structure, or induce small DNA adducts (*e.g.*, UV or methyl methanesulfonate, respectively). In general, DNA damage that leads to stalled replication elicits a common DNA damage response mediation through ataxia and Rad-related kinase (ATR; Refs. 4, 5). Thus, regardless of the nature of the treatments used to stall DNA replication, it is thought that ATR kinase initiates the activation of a protein cascade that culminates in S-phase arrest (6). ATR activation can induce phosphorylation of chk1, subsequent loss of cdc25A activity, and, ultimately, decreased initiation of new replicons (7).

Whether the DNA damage responses would be influenced by the type of agent and the amount of DNA damage is not well understood. For example, UV irradiation induces alkylation of proteins and several types of DNA damage, among which cyclobutane pyrimidine dimers and pyrimidine-pyrimidone (6-4) photoproducts are most prevalent (8). The response of cells to UV irradiation depends significantly on the amount of DNA damage (9). At low UV doses, cells predominantly undergo cell cycle arrest at the G_1-S border allowing for DNA repair, while moderate doses lead to S-phase delay and apoptosis, and high doses result in G_1 arrest (10, 11). UV-DNA adducts directly activate the ATR-chk1 pathway. However, the cellular responses at low and moderate doses are p53 dependent while those at high doses are p53 independent (10). Mitomycin C, like UV, induces varying types of DNA damage including DNA cross-links and DNA monoadducts (12). Unlike UV, low-dose mitomycin C induces cell cycle arrest at S phase and also blocks at G_2-M while increased drug concentrations result in apoptosis (13).

Recently, studies of extraordinarily cytotoxic (inhibit cell growth at ≤1 nM) DNA minor groove binding cyclopropylpyrroloindoles such as the monofunctional

Received 10/15/03; revised 2/24/04; accepted 3/9/04.

Grant support: Supported in part by NIH grants CA77491 and CA16056 (T.A. Beerman) and CA89259 and AI01686 (T. Melendy).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for Reprints: Terry A. Beerman, Department of Pharmacology and Therapeutics, Roswell Park Cancer Institute, Elm and Carlton Streets, Buffalo, NY 14263. Phone: (716) 845-3443; Fax: (716) 845-1575. E-mail: terry.beerman@roswellpark.edu

and bifunctional DNA alkylating agents adozelesin and bizelesin, respectively, have begun to reveal significant differences in their DNA damage responses. For example, adozelesin slowed cell cycle progression through S phase and blocked cells at the G_2-M interface, while bizelesin only induced a G_2-M block (14). In addition, at higher concentrations, adozelesin induced significant amounts of apoptosis, while bizelesin only caused senescence. Another highly cytotoxic monofunctional alkylator, Et743, a member of the ecteinascidin family of antibiotics, first induces a modest increase of S-phase cells and then ultimately an accumulation of cells blocked in G_2. At higher concentrations, Et743 caused time-dependent apoptosis with no further change in cell cycle (15). DNA damage responses to these highly cytotoxic DNA alkylators appear to differ between monofunctional and bifunctional DNA alkylators.

In this study, we investigated DNA damage responses with another monofunctional alkylator, hedamycin, a member of the pluramycin family of highly cytotoxic antitumor agents (16). Little is known regarding the behavior of hedamycin in mammalian cells, but based on its DNA binding mode, it would be expected to stall DNA replication and induce a cell cycle checkpoint response like other DNA damaging agents such as adozelesin and Et743. Hedamycin consists of a planar anthrapyrantrione chromophore to which are attached two aminosugar rings at one end and a bisepoxide-containing side chain at the other end (16, 17). The anthrapyrantrione chromophore intercalates the DNA duplex and threads the helix with the two aminosugar groups placed in the minor groove and the bisepoxide side chain in the major groove (18–20). It is not known whether the unique DNA binding properties of hedamycin would contribute to the type of DNA damage responses elicited in mammalian cells.

DNA damage responses, including inhibition of cell growth, macromolecular synthesis, and expression of proteins associated with interference with cell cycle progression and apoptosis, were evaluated in hedamycin-treated HCT116 cells. Changes in checkpoint proteins were measured both immediately following drug treatment and 24 h later to discern which protein are rapidly altered and whether these changes are sustained under conditions where cell cycle progression is altered.

Materials and Methods

Reagents and Cell Lines

Hedamycin was provided by National Cancer Institute (Baltimore, MD). Hedamycin stock solutions (10 mM) were prepared in water, aliquoted, and stored at −20°C. Subsequent dilutions were made in growth medium. [methyl-^{3}H]Thymidine (48 Ci/mmol), [2-^{14}C]thymidine (55 mCi/mmol), [5-^{3}H]uridine (15 mCi/mmol), and [4,5-^{3}H]leucine (100 mCi/mmol) were from Moravek Biochemicals (Brea, CA). Cell culture materials were purchased from Invitrogen (Carlsbad, CA). Other reagents were obtained from Sigma Chemical Co (St. Louis, MO). The HCT116 human colon carcinoma cell line (a gift from Dr. B. Vogelstein, Johns Hopkins Oncology Center, Baltimore, MD) was grown in McCoy's medium supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator. HeLa cells was purchased from American Type Culture Collection (Rockville, MD) and grown in DMEM supplemented with 10% fetal bovine serum at 37°C in a 5% CO_2 incubator.

Growth Inhibition Assay

Cells grown in 96-well plates were treated with drugs for 4 h and washed and incubated in fresh, drug-free medium. Following a 3-day incubation, plates were washed and cell numbers were determined by sulforhodamine B assay (21). Cell growth inhibition was calculated by comparing the absorbance of treated cells to nontreated controls.

Macromolecular Synthesis Analysis

Thymidine incorporation into acid-insoluble material was determined as described previously (22). Briefly, [^{14}C]thymidine-prelabeled cells were treated with drug for 4 h and [methyl-^{3}H]thymidine was added to a final concentration of 1 μCi/ml for the last 30 min of drug treatment. The acid-insoluble radioactivity in samples was measured by LS-3800 liquid scintillation counter. Inhibition of thymidine incorporation into cellular DNA was calculated from the ratio of ^{3}H to ^{14}C in drug-treated samples compared with nontreated controls. For measurements of RNA and protein synthesis inhibition, [5-^{3}H]uridine and [4,5-^{3}H]leucine were added to final concentrations of 1 μCi/ml for the last 30 min of the drug treatment and cells were harvested as described above.

Cell Cycle Progression Analysis

Cells were plated and treated with hedamycin as described above. After 4 h, drug was removed, plates were rinsed with PBS, and fresh medium (drug-free) was added. Cells were harvested 24 h later by trypsinization and centrifuged at 1000 rpm for 5 min. The cells were washed and fixed by the gradual addition of ice-cold 70% ethanol. Cells were stained with propidium iodide, cell cycle distribution was determined by flow cytometry analysis using a Becton Dickinson (San Jose, CA) fluorescence-activated cell sorting (FACS) Calibur flow cytometer, and data were analyzed using CellQuest software.

Terminal Deoxynucleotidyl Transferase-Mediated Nick End Labeling Assay

Cells were plated and treated with hedamycin for 4 h and incubated in fresh, drug-free medium as described above. Cells were harvested (including those cells floating in medium) and fixed with methanol for 24 h at −20°C. Cells were washed once with PBS and once with terminal deoxynucleotidyl transferase (TdT) buffer [200 mM sodium cacodylate, 25 mM Tris-HCl (pH 6.6), 0.25 mg/ml BSA] and resuspended in 50 μl TdT reaction buffer containing 10 pmol FITC-dUTP, 2.5 mM $CoCl_2$, and 12.5 units TdT. The reaction was incubated for 30 min at 37°C and stopped by adding 1 ml cold PBS containing 50 mM EDTA. After 10 min, cells were pelleted by centrifugation and resuspended in PBS. FITC incorporation was determined by using a Becton Dickinson FACS Calibur flow cytometer, and data were analyzed using CellQuest software.

Western Blot Analysis

Cells were washed twice with PBS and lysed at 4°C in a lysis buffer [50 mM HEPES (pH 7.4), 4 mM EDTA, 2 mM EGTA, 1% Triton X-100, 50 mM phenylmethylsulfonyl fluoride, 20 μg/ml leupeptine, 100 mM Na_3VO_4]. Lysates were centrifuged at 14,000 rpm for 20 min. The protein concentration in the lysate was determined by the Bio-Rad (Hercules, CA) protein assay kit. Cell lysates were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Hybond-P; Amersham Pharmacia Biotech, Piscataway, NJ). The membrane was blocked with 4% nonfat milk in Tris-buffered saline [TBS; 25 mM Tris (pH 7.4), 125 mM NaCl] for 1 h at room temperature followed by incubation with primary antibody. The antibodies used were phospho-chk1, phospho-chk2, phospho-p53-Ser^{15} and Ser^{20}, and total p53 (Cell Signaling Technology, Beverly, MA), cdc25A and cyclin E (Upstate Biotechnology, Inc., Charlottesville, VA), chk1, chk2, p21, E2F1, cdc25C, and α-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), or poly(ADP-ribose) polymerase (PARP; PharMingen, San Diego, CA). The membranes were washed thrice with TBS and incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG for 1 h. The immunoblot was washed five times with TBS and developed using an enhanced chemiluminescence Western lightning kit (Perkin-Elmer, Boston, MA). Experiments were repeated at least thrice, and loading control and corresponding protein level controls were also done by reprobing the same blots with corresponding protein antibodies.

Results

Hedamycin Inhibits Cell Growth

Cell growth was evaluated by treating cultured HCT116 cells with hedamycin for 4 h to allow sufficient time for alkylation of genomic DNA followed by sulforhodamine B assay as described in Materials and Methods. As shown in Fig. 1A, cell growth was decreased at subnanomolar concentrations. The IC_{50} (the concentration of alkylator needed to inhibit cell growth by 50%) was 0.2 nM. Because there was little hedamycin cell growth inhibition data in the literature, a second cell line (HeLa) was used to confirm that the extraordinary growth inhibitory activity is not unique to HCT116 cells (IC_{50} of HeLa cells was 0.04 nM; data not shown). To determine if cell growth inhibition is enhanced by loss of ATR, a phenomenon observed for many types of DNA damaging agents, growth inhibition was also compared in ATR-positive and ATRkd (cells lacking functional ATR due to overexpression of a kinase-dead ATR protein) human fibroblasts. Almost equivalent IC_{50} (0.1 nM) values were obtained with both (data not shown).

Hedamycin Inhibits Macromolecule Synthesis

Typically, monofunctional DNA alkylators such as adozelesin and Et743 are rapid and potent inhibitors of DNA synthesis while showing substantially less activity at blocking RNA and protein synthesis (23). To examine for such immediate effects of hedamycin on these biological processes, the ability of short-term treatments to block DNA synthesis was determined. Pulse labeling of cells with [^{3}H]thymidine following a 4 h treatment with 0.1 nM hedamycin showed that DNA synthesis was inhibited by 50% (Fig. 1B). RNA synthesis was also inhibited by hedamycin, but the concentration needed to inhibit by 50% was about 10-fold higher (*i.e.*, 1 nM) than that required for DNA synthesis inhibition. Protein synthesis was essentially unaffected by similar concentrations of hedamycin (Fig. 1B) and no inhibition was observed even when cells were exposed to high hedamycin concentrations (5 nM; data not shown).

Hedamycin Inhibition of Cell Cycle Progression Induces Substantial S-Phase Arrest

Hedamycin was examined for its effects on HCT116 cell cycle progression. Incubation of HCT116 cells with hedamycin for 4 h followed by removal of the drug and further incubation for 24 h resulted in concentration-dependent changes in cell cycle progression (Fig. 2A). Cells treated with 0.1 nM hedamycin accumulated in G_2 (44%) compared with a control value of 24%, but the block was temporary

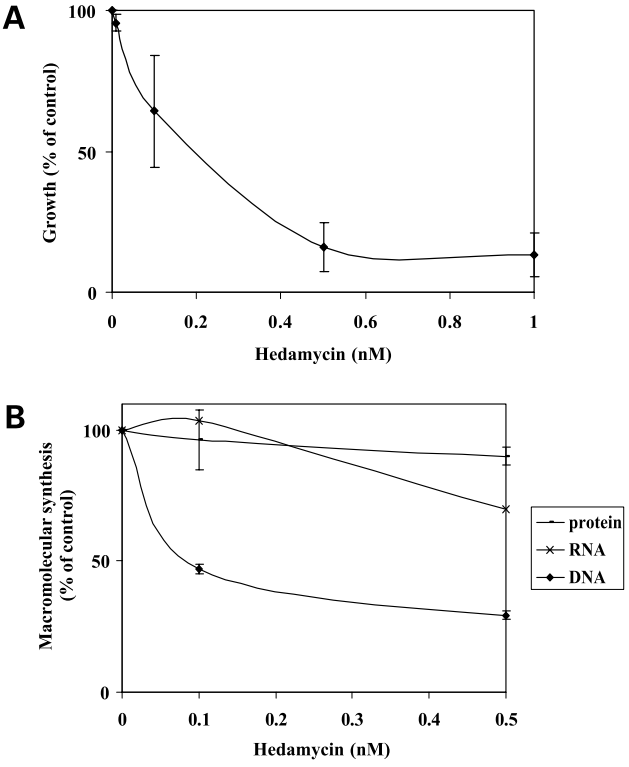

Figure 1. **A,** hedamycin inhibits cell growth. Proliferating cells were treated with hedamycin for 4 h, washed, incubated in drug-free medium for another 3 days, and counted. Results are expressed as a percentage of untreated control cells. Average of three individual values for each drug concentration. *Points,* mean; *bars,* SE (n = 3). **B,** hedamycin strongly inhibits DNA synthesis. HCT116 cells cultured in [^{14}C]thymidine-containing medium for 48 h were incubated with hedamycin for 4 h and pulse labeled with [^{3}H]thymidine 30 min before harvest as described in Materials and Methods. The ratios of DNA incorporation of $^3H/^{14}C$ were calculated and expressed as a percentage of incorporation by untreated control cells. *Points,* mean; *bars,* SE (n = 3). For analysis of RNA and protein synthesis, cells were pulse labeled with [^{3}H]uridine or [^{3}H]leucine 30 min before harvest.

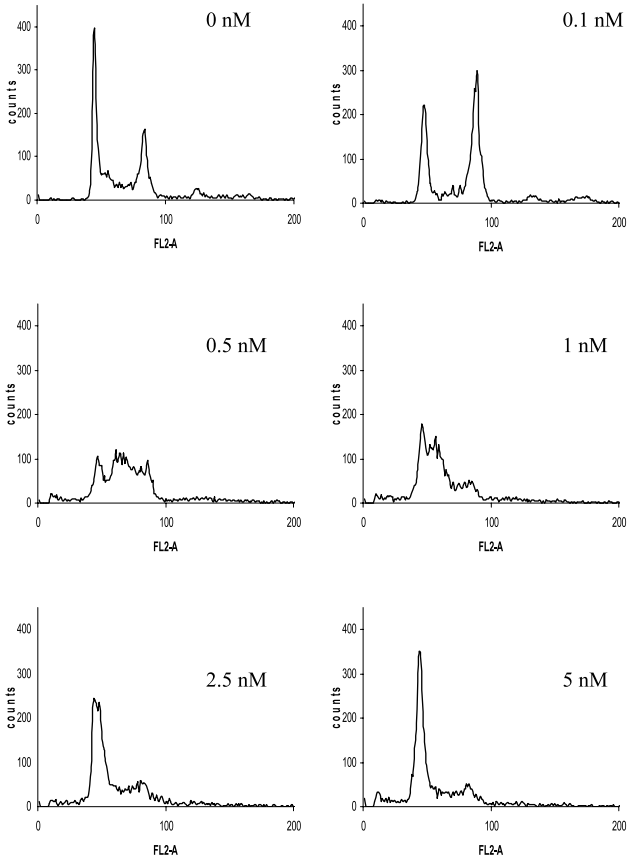

Figure 2. Dose-dependent drug effects on cell cycle progression. HCT116 cells were treated with hedamycin for 4 h and further incubated in drug-free medium for 24 h. Cells were harvested, fixed, and stained with propidium iodide. DNA content was analyzed using FACS and a commercially available software WinList.

because it was reversed 48 h after drug removal (data not shown). In contrast, S-phase cell arrest was observed following hedamycin treatments of 0.5 and 1 nM (43%) *versus* control cells (21%). Unlike the transient G_2 block, cells remained trapped in S phase after 48 h (data not shown). Somewhat higher concentrations of hedamycin (2.5 and 5 nM) caused persistent G_1 arrest (46% and 59% *versus* 34% in the control) without a detectable sub-G_1 cell population and cell loss. Again, cell cycle changes were sustainable 48 h after release (data not shown). Similar results were obtained with other mammalian cell lines including ATR-deficient cells (data not shown).

Hedamycin Interference with Cell Cycle Progression Was Accompanied by Changes in Several Checkpoint Proteins Known to be Associated with S-Phase Arrest

Significant changes in cell cycle distribution were seen when cells were incubated in fresh (alkylator-free) medium for 24 h following a 4 h treatment. To determine what checkpoint proteins were associated with these cell cycle alterations, lysates from hedamycin-treated cells were analyzed by Western blotting. As shown in Fig. 3, activation of p53 via phosphorylation of Ser^{15} increased significantly at all concentrations from 0.5 nM where cells arrest in S or G_1, while with 0.1 nM, which caused transient accumulation in G_2, no signal increase was detected. Subsequent induction of the p53 downstream target p21, which usually mediates G_1 or G_2-M arrest when DNA replication forks are stalled, was also observed (24, 25). As shown in Fig. 3, p21 was not induced in G_1-arrested cells (5 nM) and was only weakly induced with 0.1 or 2.5 nM concentrations that cause cells to arrest in G_2 or G_1-S. p21 was overexpressed only by alkylator treatments that resulted in S-phase arrest (0.5 and 1 nM).

Treatments with DNA damaging agents that cause S-phase arrest are also known to be associated with phosphorylation/activation of chk1 and chk2 kinases (26). Figure 3 shows that only chk1 phosphorylation was detected at the lowest hedamycin concentration (0.1 nM). However, both chk1 and chk2 were phosphorylated at 0.5–2.5 nM hedamycin, which caused cells to strongly arrest in S phase or at the G_1-S boundary. In contrast, phosphorylation of both chk proteins was reduced at 5 nM treatments where cells were arrested in G_1.

Cdc25A Is Reduced in Parallel with S-Phase Arrest while E2F1 and Cyclin E Are Induced

Activation of chk1 protein associated with DNA damage-induced S-phase arrest is accompanied by loss of cdc25A, a protein involved in stimulating initiation of DNA replication (27, 28). When cells were treated with hedamycin for 4 h and further incubated in alkylator-free medium for 24 h, a condition that induced significant S-phase arrest, cdc25A expression was markedly reduced (Fig. 4). At the same time, cdc25C, a protein that is decreased in G_2-arrested cells (29), was unaffected. Two other proteins known to be required for G_1-S transitions, E2F1 and cyclin E, were also tested for their sustained expression during hedamycin-induced S-phase arrest (30, 31). Cyclin E, which is required for G_1-S transitions, was found to be elevated in cells that were blocked primarily in S phase (0.5–2.5 nM) but was reduced at the highest hedamycin concentration (5.0 nM), which resulted in almost complete cell cycle arrest. Similarly, E2F1 expression is associated with entry of cells

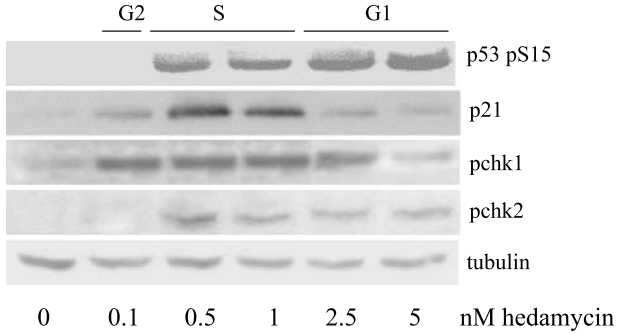

Figure 3. Hedamycin induced sustained checkpoint protein responses. HCT116 cells were treated with hedamycin as described in Fig. 2. Cell lysates were prepared and analyzed by SDS-PAGE and Western blotting. Blots were probed with antibodies against phospho-p53-Ser^{15} (*p53 pS15*), p21, phospho-chk1 (*pchk1*), phospho-chk2 (*pchk2*), and tubulin. *Top of the Western blots,* cell cycle arrest positions.

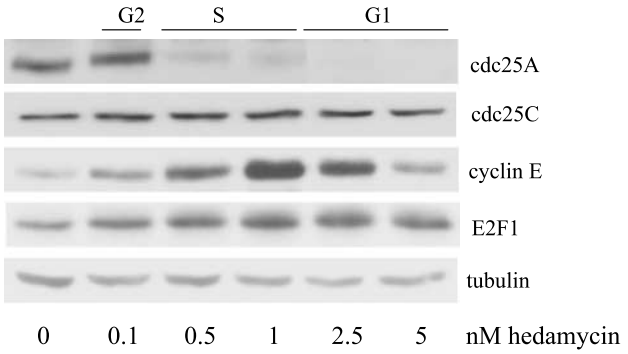

Figure 4. Changes in S-phase-related proteins after hedamycin treatment. Hedamycin treatment and Western blot analysis were as described in Fig. 3. Western blots were probed with antibodies against cdc25A, cdc25C, cyclin E, E2F1, and tubulin. *Top of the Western blots,* cell cycle arrest positions.

into S phase and is enhanced at hedamycin concentrations that induce increased S-phase arrest. Unlike cyclin E, E2F1 levels remained elevated even when cells were prevented from cycling at the highest hedamycin level (5.0 nM).

Checkpoint Proteins Are Rapidly Activated by Hedamycin

Several of the key DNA response proteins including chk1, chk2, and p53, the alteration of which was sustained under conditions where hedamycin induced inhibition of cell cycle progression (24 h after drug removal), are also known to be quickly phosphorylated following introduction of DNA damage. Short-term hedamycin-induced activation of DNA damage responses were evaluated by incubating HCT116 cells with drug for 4 h after which cell lysates were prepared and analyzed by Western blotting. Figure 5A shows that increasing hedamycin concentrations cause increased levels of phosphorylated p53. As little as 0.1 nM hedamycin is sufficient to induce activation of p53 through enhancement of Ser^{15} phosphorylation, and the phosphorylation level approaches a plateau at concentrations above 0.5 nM. An increase in total p53 protein levels was observed in parallel with Ser^{15} phosphorylation, although Ser^{20} phosphorylation (data not shown) and p21 induction were undetectable.

Chk1 and chk2 phosphorylation are also rapidly stimulated as part of a checkpoint response following DNA damage. As shown in Fig. 5A, chk1 phosphorylation was first detected at 0.1 nM hedamycin and reached a peak at 1 nM before decreasing at higher concentrations (2.5 and 5 nM). In comparison, chk2 phosphorylation was induced at drug concentrations above 0.1 nM and decreased in parallel with chk1.

Cdc25A is a cyclin-dependent kinase phosphatase, which controls the G_1-S cell cycle transition and would be expected to decrease under treatment conditions that would result in blocking the G_1-S transition. At low and moderate concentrations of hedamycin, there was little decrease in cdc25A (0.1 nM; Fig. 5A), while an almost complete lose was observed at higher concentrations, which would prohibit cell entry into S phase (2.5 and 5 nM; Fig. 5A).

A time course depicting the speed with which these protein changes can occur in response to hedamycin-induced DNA damage was determined by incubating cells under conditions that would induce strong S-phase arrest (0.8 nM hedamycin) for various times (15 min to 4 h). The concentration chosen induced similar phosphorylation levels for p53, chk1, and chk2 after 4 h. As shown in Fig. 5B, phosphorylation of p53 Ser^{15}, chk1, and chk2 was observed within 30 min treatment. The phosphorylation level of p53 Ser^{15} increased in a time-dependent manner for 180 min, while phosphorylation of chk1 and chk2 was complete after 30 min. Levels of cdc25A increased within 15 min and decreased between 30 and 120 min. These results demonstrate that several checkpoint proteins are very rapidly activated following DNA damage induction by hedamycin.

PARP Cleavage and TdT-Mediated Nick End Labeling–Positive Staining Are Induced after Exposure to Moderate but not High Concentrations of Hedamycin

The FACS profiles showed only minor increases in the sub-G_1 population under conditions that blocked cell cycle progression (0.5–5 nM; see Fig. 2A), indicating that cells showing the advanced stages of apoptosis were limited. To determine whether any apoptotic-associated events were being induced, hedamycin-treated cells were assayed for TdT-mediated nick end labeling (TUNEL) staining as a result of apoptosis-related DNA degradation. As shown in Fig. 6A, TUNEL-positive cells were easily detected 24 h after exposure to moderate concentrations of hedamycin (0.5–1 nM), which were shown to induce S-phase arrest. However, at higher alkylator levels (2.5 and 5 nM), the

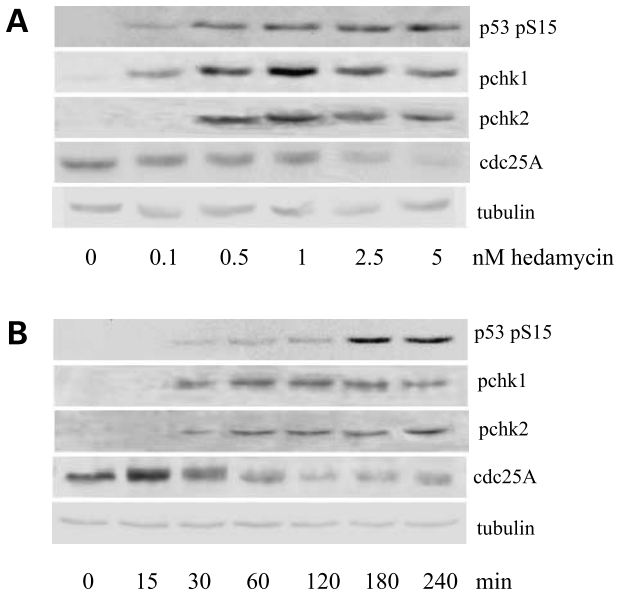

Figure 5. Western blots showing checkpoint activation during short-term hedamycin exposure. **A,** HCT116 cells were incubated with the indicated concentrations of hedamycin for 4 h. **B,** cells were treated with 0.8 nM hedamycin for the indicated times. Hedamycin treatment and Western blot analysis were as described in Fig. 3. Blots were probed with antibodies against phospho-p53-Ser^{15} (*p53 pS15*), phospho-chk1 (*pchk1*), phospho-chk2 (*pchk2*), cdc25A, and tubulin.

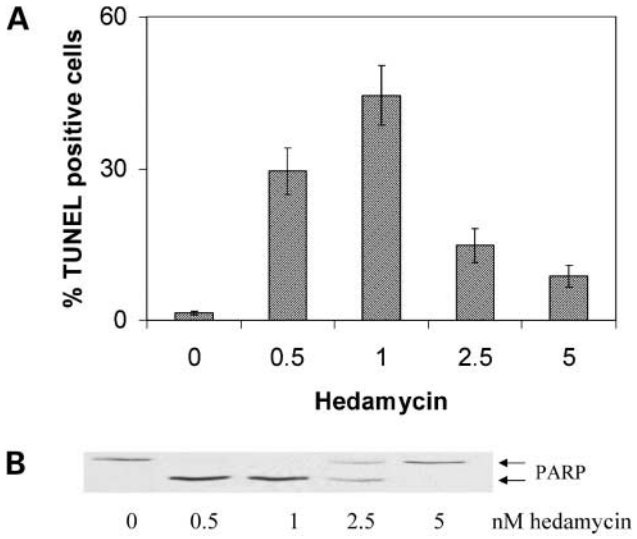

Figure 6. Induction of apoptotic markers by hedamycin. HCT116 cells were exposed to the indicated concentrations of hedamycin for 4 h, washed, and incubated in drug-free medium for another 24 h. **A,** apoptosis was determined by TUNEL assay and analyzed by FACS. Results are expressed as the percentage TUNEL-positive cells. Average of three individual experiments. *Columns,* mean; *bars,* SE (n = 3). **B,** hedamycin treatment and Western blot analysis were as described in Fig. 3 and analyzed for another apoptotic marker, PARP cleavage. Blots were probed with PARP antibody.

TUNEL-positive cell signal was substantially reduced. After 48 h, the percentage of the TUNEL-positive cells in each treatment remained essentially unchanged as compared with 24 h (data not shown).

Because the TUNEL signal could also reflect hedamycin-induced DNA breaks by other mechanisms, a second marker of apoptotic signaling, loss of intact PARP protein and appearance of a specific PARP degradation product was examined. Figure 6B is a Western blot showing PARP, which is proteolytically cleaved when cells are becoming apoptotic. Nearly all of the cellular PARP was cleaved at moderate hedamycin concentrations (0.5–1 nM) but only partially cleaved at 2.5 nM. Increasing the drug concentration to 5 nM resulted in complete loss of PARP cleavage. Changes in the amount of PARP cleavage were concordant with cells showing positive TUNEL staining. Overall, the results show that induction of apoptotic signaling events occurs predominantly with treatments that result in strong S-phase arrest.

Discussion

This study explored cellular responses to hedamycin, a highly cytotoxic monofunctional DNA alkylator that both covalently binds DNA through N7 of guanine in the major groove and through threading intercalation that involves both DNA grooves. Hedamycin was found to alter HCT116 cell cycle distribution in a concentration-dependent manner that resulted in a strong S-phase arrest under moderate treatment conditions. At very low concentrations, some cells transiently accumulated in G_2, while moderate concentrations (0.5–1 nM) resulted in most cells accumulating in S phase. At higher concentrations (≥2.5 nM), cells were unable to cycle.

The S-phase block was reminiscent of the DNA alkylating drug adozelesin, which, at a comparable cytotoxicity to hedamycin in HCT116 cells (twice its D_{10}, the concentration needed to reduce clonogenic survival by 90%), also induced a S-phase slowdown, albeit to a much lower extent (14). However, unlike adozelesin, which at higher drug concentrations (>12 times its D_{10}), induces a substantial increase in the sub-G_1 population, hedamycin at 12 times its D_{10} (5 nM) does not. The inability of a DNA damaging agent to drive cells toward apoptosis under extreme treatment conditions (>50 times its D_{10}) was also observed for bizelesin, a bifunctional DNA alkylator. At the same time, bizelesin, unlike hedamycin, was unable to block cells in S phase and induced only G_2 arrest. Indeed, the type of DNA damage that elicits a cell cycle progression inhibitory response that is most similar to hedamycin is UV radiation, a treatment that does not induce alkylation but rather creates pyrimidine dimers and various other photogenerated products (8). Moderate UV treatment induces S-phase arrest and apoptosis, albeit not to the extent observed with hedamycin, while more severe treatments result in G_1 arrest (10).

While the nature of hedamycin-induced cell cycle arrest is distinct from what is observed with certain DNA alkylators, the activation of associated cell cycle checkpoint proteins is consistent with profiles observed for DNA damage leading to an inhibition of initiation of DNA replication. First, the general stress response protein p53 is activated via phosphorylation of Ser^{15} (Ser^{20} was not rapidly phosphorylated; data not shown) at hedamycin levels that induce significant S-phase arrest as well as a G_1 block (Figs. 3 and 5). As expected, expression of p21, a downstream target of p53, was also enhanced but only under conditions where cells arrest in S phase.

Cells cannot arrest in S phase for too long or replication forks can collapse leading to cell death (32). Consistent with this perception, maximal apoptotic signaling events were observed when cells were trapped in S phase and coincided with induction of p53 and its major downstream target p21. p21 is known to protect cells from apoptosis induced by a variety of cellular stresses including DNA damaging agents like doxorubicin, IR, and camptothecin (33–35). However, its antiapoptotic effects are believed to rely on induction of G_1 or G_2 arrest but not S phase (36–38). Interestingly, the highest levels of apoptosis-associated events (*e.g.*, TUNEL signaling and PARP cleavage) were observed when p21 levels were elevated; hence, p21 does not appear to play a protective role against hedamycin-induced apoptotic signaling status (Figs. 3 and 6). On the other hand, for adozelesin, p21 levels are decreased when cells become apoptotic (14).

Hedamycin induction of high levels of p21 during S-phase arrest was also found to be associated with proliferating cell nuclear antigen (PCNA) based on coimmunoprecipitation experiments (data not shown). As a

consequence, p21 might limit PCNA availability for DNA repair and replication so that cells could not complete S phase. Although the role of p21 in regulating DNA repair is still controversial, p21 could regulate nucleotide excision repair and base excision repair by binding with PCNA and thus sequester PCNA to impede its recycling resulting in failure of repair (39–41). It was reported previously that after DNA damage in a p21-deficient cell line, PCNA retention at repair sites was increased (42).

While both chk1 and chk2 protein kinases are involved in the early stages of induction of cell cycle checkpoints in response to DNA damage, the former is associated with damage that leads to stalling of DNA replication while the later is induced in response to DNA double-strand breaks (5, 6). Thereafter, when ongoing replication forks collide with damaged DNA, an intra-S-phase checkpoint is initiated via phosphorylation/activation chk1 or chk2 proteins, which leads to stabilization of incomplete replication forks and retardation of S-phase progression and allows for additional time for DNA repair (43). Hedamycin induces a bulky DNA adduct, making it similar to DNA damage that leads to stalled replication such as that induced by UV, consistent with the greater sensitivity of both short-term and long-term chk1 phosphorylation compared with chk2 (Figs. 3 and 5). Hedamycin at higher concentrations also increased both short-term and sustained chk2 phosphorylation (Figs. 3 and 5), suggesting that low levels of DNA double-strand breaks might ultimately be produced.

Chk1 is known to activate cell cycle checkpoint by mediating degradation of cdc25A resulting in loss of cdk2/cyclin E activation and preventing G_1-S transition (44, 45). Although chk1 can activate S-phase checkpoint by promoting the degradation of cdc25A, the concentration-dependent and time-dependent decrease of cdc25A (Figs. 3A and 5) indicates that cells were able to entry S phase when they first encounter hedamycin-induced DNA lesions.

Hedamycin-enhanced sustained E2F1 expression (Fig. 4) might also contribute to the apoptotic signaling events that were seen in S-phase-arrested cells. Treatments such as γ-irradiation or cisplatin induce cell death via apoptosis involving E2F1, which serves to activate proapoptotic or suppress antiapoptotic genes (46–48). Alternatively, E2F1 may enhance the apoptotic function of p53 (49). Recent studies showed that expression of E2F1 increases p53 accumulation, coinciding with the induction of apoptosis (50). Another study suggested that E2F1 forms a complex with p53 following DNA damage and converts latent p53 into an active form leading to apoptosis (51).

Throughout the analysis of cellular responses to hedamycin, it was noticed that not only were expression of damage response proteins such as chk1 more evident during S-phase arrest but also were indicators of apoptotic signaling events. The increases in these markers for apoptotic stress at moderate concentrations and decreases at higher concentrations were similar to that observed with cisplatin (52). However, hedamycin-induced apoptotic signaling was associated with a S-phase block while cisplatin caused a G_1 block. In contrast, many DNA damaging agents such as camptothecin, Adriamycin, and monofunctional alkylators adozelesin and Et743 typically induce a concentration-dependent increase in apoptosis and a G_2 block (53–56).

At the highest hedamycin concentration tested (5 nM), cells were permanently arrested in G_1. Both chk proteins can activate a G_1 checkpoint via p53-dependent or p53-independent pathways resulting in induction of p21 (57). Although p53 Ser^{15} phosphorylation was detected, its downstream target p21 was not present in G_1-arrested cells. On the other hand, hedamycin-induced G_1 arrest possibly resulted from permanent loss of cdc25A, which disappeared within 4 h of drug treatment (Fig. 5A).

Whether due to high levels of DNA lesions or their physical characteristics, the cells appear unable to mount a complete apoptotic response, but rather the combination of direct inhibition of DNA replication and blocking of cell cycle progression appears to hold the cells in G_1. The cell numbers also did not change and cells remained attached to the plate with intact morphology even when treatment times were extended to 48 h.

Overall, many of the damage responses of cells to hedamycin are dissimilar to many types of DNA damaging drugs and unexpectedly more similar to those induced by UV. Among these similarities are a failure to show strong G_2 arrest but rather a block at G_1-S, induction of chk1 phosphorylation before chk2, induction of rapid cdc25A degradation, an increase in cyclin E levels at moderate doses, induction of apoptosis, and induction of an association of p21 and PCNA. However, hedamycin also differs from UV in several significant aspects. The most notable differences are as follows: hedamycin induces an extremely strong S-phase arrest while UV induces only a partial S-phase slowdown; the cellular effects of hedamycin are p53 independent while UV-induced cell cycle effects and apoptosis are p53 dependent at low and moderate doses and p53 independent G_1 arrest at high doses; p21 association with PCNA coincides with S-phase arrest after hedamycin treatment but with G_1 arrest following UV irradiation; and hedamycin induces permanent loss of cdc25A and not the transient loss observed after UV irradiation.

This study was the first to examine cellular DNA damage responses to the highly cytotoxic monofunctional DNA alkylator, hedamycin. In comparison with many types of DNA damaging agents, which induce strong G_2 arrest, hedamycin blocked cells in S phase or at the G_1-S border. Moreover, unlike a typical DNA alkylator such as adozelesin, treatments at high concentrations do not induce sub-G_1 cell populations associated with apoptosis. It is of interest to understand why cells arrest so strongly in S phase after hedamycin treatment. Detailed mechanism studies including the role of ATR/chk1 in hedamycin-induced S-phase arrest are currently underway. While preliminary data show that interference of cell cycle progression by hedamycin is independent of ATR, further studies are needed to define the role of ATR or other phosphatidylinositol 3-kinase-related kinase in the regulation of cellular responses to hedamycin-DNA adducts.

Additionally, hedamycin like adozelesin appears to induce replication stalling by blocking initiation, but unlike the later, which occurs in *trans*, hedamycin uses a *cis*-acting mechanism (Tu and Beerman, unpublished observation; Ref. 58). Apparently, hedamycin, which is one of the most powerful monofunctional alkylator inhibitors of DNA replication, may be dual acting by blocking S-phase transition by both induction of checkpoint responses and a direct effect on DNA synthesis.

Acknowledgments

We thank Drs. Mary M. McHugh and Athena Lin for the advice and comments on the manuscript.

References

1. Elledge SJ. Cell cycle checkpoints: preventing an identity crisis. Science 1996;274:1664 – 72.

2. Weinert T. DNA damage and checkpoint pathways: molecular anatomy and interactions with repair. Cell 1998;94:555 – 8.

3. Matsuoka S, Huang M, Elledge SJ. Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. Science 1998;282:1893 – 7.

4. Zhao H, Piwnica-Worms H. ATR-mediated checkpoint pathways regulate phosphorylation and activation of human Chk1. Mol Cell Biol 2001;21:4129 – 39.

5. Shiloh Y. ATM and ATR: networking cellular responses to DNA damage. Curr Opin Genet Dev 2001;11:71 – 7.

6. Abraham RT. Cell cycle checkpoint signaling through the ATM and ATR kinases. Genes Dev 2001;15:2177 – 96.

7. Bartek J, Lukas J. Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell 2003;3:421 – 9.

8. Brash DE, Rudolph JA, Simon JA, et al. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. Proc Natl Acad Sci USA 1991;88:10124 – 8.

9. Decraene D, Agostinis P, Bouillon R, Degreef H, Garmyn M. Insulin-like growth factor-1-mediated AKT activation postpones the onset of ultraviolet B-induced apoptosis, providing more time for cyclobutane thymine dimer removal in primary human keratinocytes. J Biol Chem 2002;277:32587 – 95.

10. Chang D, Chen F, Zhang F, McKay BC, Ljungman M. Dose-dependent effects of DNA-damaging agents on p53-mediated cell cycle arrest. Cell Growth & Differ 1999;10:155 – 62.

11. Ljungman M, Zhang F, Chen F, Rainbow AJ, McKay BC. Inhibition of RNA polymerase II as a trigger for the p53 response. Oncogene 1999;18: 583 – 92.

12. Abbas T, Olivier M, Lopez J, et al. Differential activation of p53 by the various adducts of mitomycin C. J Biol Chem 2002;277: 40513 – 9.

13. Kang SG, Chung H, Yoo YD, Lee JG, Choi YI, Yu YS. Mechanism of growth inhibitory effect of mitomycin-C on cultured human retinal pigment epithelial cells: apoptosis and cell cycle arrest. Curr Eye Res 2001; 22:174 – 81.

14. Cao PR, McHugh MM, Melendy T, Beerman T. The DNA minor groove-alkylating cyclopropylpyrroloindole drugs adozelesin and bizelesin induce different DNA damage response pathways in human colon carcinoma HCT116 cells. Mol Cancer Ther 2003;2:651 – 9.

15. Gajate C, An F, Mollinedo F. Differential cytostatic and apoptotic effects of ecteinascidin-743 in cancer cells. Transcription-dependent cell cycle arrest and transcription-independent JNK and mitochondrial mediated apoptosis. J Biol Chem 2002;277:41580 – 9.

16. Bradner WT, Heinemann B, Gourevitch A. Hedamycin, a new antitumor antibiotic. II. Biological properties. Antimicrob Agents Chemother 1966;6:613 – 8.

17. Schmitz H, Crook KE Jr, Bush JA. Hedamycin, a new antitumor antibiotic. I. Production, isolation, and characterization. Antimicrob Agents Chemother 1966;6:606 – 12.

18. Murray V, Moore AG, Matias C, Wickham G. The interaction of hedamycin and DC92-B in a sequence selective manner with DNA in intact human cells. Biochim Biophys Acta Gene Struct Expr 1995;1261: 195 – 200.

19. Hansen M, Yun S, Hurley L. Hedamycin intercalates the DNA helix and, through carbohydrate-mediated recognition in the minor groove, directs N7-alkylation of guanine in the major groove in a sequence-specific manner. Chem Biol 1995;2:229 – 40.

20. Cairns MJ, Murray V. The DNA sequence specificity of hedamycin damage determined by ligation-mediated PCR and linear amplification. Biochem Mol Biol Int 1998;46:267 – 75.

21. Papazisis KT, Geromichalos GD, Dimitriadis KA, Kortsaris AH. Optimization of the sulforhodamine B colorimetric assay. J Immunol Methods 1997;208:151 – 8.

22. McHugh MM, Woynarowski JM, Gawron LS, Otani T, Beerman, TA. Effects of the DNA-damaging enediyne C-1027 on intracellular SV40 and genomic DNA in green monkey kidney BSC-1 cells. Biochemistry 1995;34:1805 – 14.

23. Bhuyan BK, Smith KS, Adams EG, Wallace TL, Von Hoff DD, Li LH. Adozelesin, a potent new alkylating agent: cell-killing kinetics and cell-cycle effects. Cancer Chemother Pharmacol 1992;30:348 – 54.

24. Waldman T, Kinzler KW, Vogelstein B. p21 is necessary for the p53-mediated G_1 arrest in human cancer cells. Cancer Res 1995;55: 5187 – 90.

25. Bunz F, Dutriaux A, Lengauer C, et al. Requirement for p53 and p21 to sustain G_2 arrest after DNA damage. Science 1998;282:1497 – 501.

26. Boddy MN, Furnari B, Mondesert O, Russell P. Replication checkpoint enforced by kinases Cds1 and Chk1. Science 1998;280:909 – 12.

27. Galaktionov K, Lee AK, Eckstein J, et al. CDC25 phosphatases as potential human oncogenes. Science 1995;269:1575 – 7.

28. Mailand N, Falck J, Lukas C, et al. Rapid destruction of human Cdc25A in response to DNA damage. Science 2000;288:1425 – 9.

29. Peng CY, Graves PR, Thoma RS, Wu ZQ, Shaw AS, Piwnica-Worms H. Mitotic and G_2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science 1997;277: 1501 – 5.

30. Dyson N. The regulation of E2F by pRB-family proteins. Genes Dev 1998;12:2245 – 62.

31. Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM, Pagano M. Human cyclin E, a nuclear protein essential for the G_1-to-S phase transition. Mol Cell Biol 1995;15:2612 – 24.

32. Donaldson AD, Blow JJ. DNA replication: stable driving prevents fatal smashes. Curr Biol 2001;11:R979 – 82.

33. Waldman T, Zhang Y, Dillehay L, et al. Cell-cycle arrest *versus* cell death in cancer therapy. Nat Med 1997;3:1034 – 6.

34. Martinez LA, Yang J, Vazquez ES, et al. M. p21 modulates threshold of apoptosis induced by DNA-damage and growth factor withdrawal in prostate cancer cells. Carcinogenesis 2002;23:1289 – 96.

35. Han Z, Wei W, Dunaway S, et al. Role of p21 in apoptosis and senescence of human colon cancer cells treated with camptothecin. J Biol Chem 2002;277:17154 – 60.

36. Gervais JL, Seth P, Zhang H. Cleavage of CDK inhibitor p21(Cip1/Waf1) by caspases is an early event during DNA damage-induced apoptosis. J Biol Chem 1998;273:19207 – 12.

37. Gorospe M, Cirielli C, Wang X, Seth P, Capogrossi MC, Holbrook NJ. p21(Waf1/Cip1) protects against p53-mediated apoptosis of human melanoma cells. Oncogene 1997;14:929 – 35.

38. Levkau B, Koyama H, Raines EW, et al. Cleavage of p21Cip1/Waf1 and p27Kip1 mediates apoptosis in endothelial cells through activation of Cdk2: role of a caspase cascade. Mol Cell 1998;1:553 – 63.

39. Pan ZQ, Reardon JT, Li L, et al. Inhibition of nucleotide excision repair by the cyclin-dependent kinase inhibitor p21. J Biol Chem 1995;270: 22008 – 16.

40. Waga S, Hannon GJ, Beach D, Stillman B. The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. Nature 1994;369:574 – 8.

41. Li R, Waga S, Hannon GJ, Beach D, Stillman B. Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. Nature 1994;371:534 – 7.

42. Stivala LA, Riva F, Cazzalini O, Savio M, Prosperi E. p21(waf1/cip1)-null human fibroblasts are deficient in nucleotide excision repair downstream the recruitment of PCNA to DNA repair sites. Oncogene 2001; 20:563 – 70.

43. Feijoo C, Hall-Jackson C, Wu R, et al. Activation of mammalian Chk1 during DNA replication arrest: a role for Chk1 in the intra-S phase checkpoint monitoring replication origin firing. J Cell Biol 2001;154: 913 – 23.

44. Furstenthal L, Kaiser BK, Swanson C, Jackson PK. Cyclin E uses Cdc6 as a chromatin-associated receptor required for DNA replication. J Cell Biol 2001;152:1267 – 78.

45. Kilbey A, Stephens V, Bartholomew C. Loss of cell cycle control by deregulation of cyclin-dependent kinase 2 kinase activity in Evi-1 transformed fibroblasts. Cell Growth & Differ 1999;10:601 – 10.

46. Lin WC, Lin FT, Nevins JR. Selective induction of E2F1 in response to DNA damage, mediated by ATM-dependent phosphorylation. Genes Dev 2001;15:1833 – 44.

47. Nahle Z, Polakoff J, Davuluri RV, et al. Direct coupling of the cell cycle and cell death machinery by E2F. Nat Cell Biol 2002;4:859 – 64.

48. Ginsberg D. E2F1 pathways to apoptosis. FEBS Lett 2002;529:122.

49. Blattner C, Sparks A, Lane D. Transcription factor E2F-1 is upregulated in response to DNA damage in a manner analogous to that of p53. Mol Cell Biol 1999;19:3704 – 13.

50. Wu X, Levine AJ. p53 and E2F-1 cooperate to mediate apoptosis. Proc Natl Acad Sci USA 1994;91:3602 – 6.

51. Hsieh JK, Yap D, O'Connor DJ, et al. Novel function of the cyclin A binding site of E2F in regulating p53-induced apoptosis in response to DNA damage. Mol Cell Biol 2002;22:78 – 93.

52. Cummings BS, Schnellmann RG. Cisplatin-induced renal cell apoptosis: caspase 3-dependent and -independent pathways. J Pharmacol Exp Ther 2002;302:8 – 17.

53. Zhang ZW, Patchett SE, Farthing MJ. Topoisomerase I inhibitor (camptothecin)-induced apoptosis in human gastric cancer cells and the role of wild-type p53 in the enhancement of its cytotoxicity. Anticancer Drugs 2000;11:757 – 64.

54. Zhang XW, Qing C, Xu B. Apoptosis induction and cell cycle perturbation in human hepatoma hep G_2 cells by 10-hydroxycamptothecin. Anticancer Drugs 1999;10:569 – 76.

55. Fornari FA Jr, Jarvis DW, Grant S, et al. Growth arrest and non-apoptotic cell death associated with the suppression of c-*myc* expression in MCF-7 breast tumor cells following acute exposure to doxorubicin. Biochem Pharmacol 1996;51:931 – 40.

56. Ababou M, Dutertre S, Lécluse Y, Onclercq R, Chatton B, Amor-Guéret M. ATM-dependent phosphorylation and accumulation of endogenous BLM protein in response to ionizing radiation. Oncogene 2000;19:5955 – 63.

57. Bartek J, Lukas J. Mammalian G_1- and S-phase checkpoints in response to DNA damage. Curr Opin Cell Biol 2001;13:738 – 47.

58. Cobuzzi RJ Jr, Burhans WC, Beerman TA. Inhibition of initiation of simian virus 40 DNA replication in infected BSC-1 cells by the DNA alkylating drug adozelesin. J Biol Chem 1996;271:19852 – 9.