Mitomycin C and doxorubicin elicit conflicting signals by causing accumulation of cyclin E prior to p21$^{WAF1/CIP1}$ elevation in human hepatocellular carcinoma cells

SUN-YOUNG CHOI[1,5,6*], YAN NAN SHEN[1*], SEON RANG WOO[1], MIYOUNG YUN[1], JEONG-EUN PARK[1], YEUN-JIN JU[1], JAEMIN JEONG[1], HYUN-JIN SHIN[1], HYUN-YOO JOO[1], EUN-RAN PARK[1], JUNG-KEE LEE[2], SANG HOON KIM[3], MYUNG-HAING CHO[4], IN-SOO KONG[5] and KEE-HO LEE[1]

[1]Division of Radiation Cancer Research, Korea Institute of Radiological & Medical Sciences, Seoul 139-706; [2]Department of Life Science & Genetic Engineering, Paichai University, Daejeon 302-735; [3]Department of Biology, Kyung Hee University, Seoul 130-701; [4]Laboratory of Toxicology, College of Veterinary Medicine and the Research Institute for Veterinary Science, Seoul National University, Seoul 151-74-2; [5]Department of Biotechnology, Pukyong National University, Busan 608-737; [6]Department of Biological Analysis, Chemical Defense Research Institute, Seoul 137-180, Republic of Korea

Received June 16, 2011; Accepted July 25, 2011

DOI: 10.3892/ijo.2011.1184

Abstract. Proteins involved in the G_1 phase of the cell cycle are aberrantly expressed, sometimes in mutated forms, in human cancers including human hepatocellular carcinoma. Upon attack by a DNA-damaging anticancer drug, a cell arrests at the G_1 phase; this is a safety feature prohibiting entry of DNA-damaged cells into S-phase. p21$^{WAF1/CIP1}$ prevents damaged cells from progressing to the next cell cycle. Here, we show that, in response to mitomycin C and doxorubicin, human hepatocellular carcinoma cells generate conflicting signals, mediated by cyclin E and p21$^{WAF1/CIP1}$, which respectively accelerates and represses cell cycle transition. Exposure to these anticancer drugs led to rapid accumulation of cyclin E in both p53-proficient HepG2 and p53-deficient Hep3B cells. Such anticancer drug-induced cyclin E accumulation influenced the G_1-S-phase transition, but not DNA fragmentation-mediated death. In p53-proficient HepG2 cells, accumulation of cyclin E was followed by an increase in the level of p53-dependent p21$^{WAF1/CIP1}$, thereby inhibiting further the G_1-S-phase transition. Sublethal drug concentrations also induced rapid accumulation of cyclin E, but p21$^{WAF1/CIP1}$ accumulation was delayed, further facilitating the G_1-S-phase transition. Eventually, most cells arrested in G_2/M. Thus, mitomycin C- or doxorubicin-induced conflicting signals, mediated by cyclin E and p21$^{WAF1/CIP1}$, are in play in human hepatocellular carcinoma cells. Damaged G_1 cells either immediately enter S-phase, or do not do so at all, depending on the extent of DNA damage.

Correspondence to: Dr Kee-Ho Lee, Division of Radiation Cancer Research, Korea Institute of Radiological & Medical Sciences, 215-4 Gongneung-dong, Nowon-Ku, Seoul 139-706, Republic of Korea
E-mail: khlee@kirams.re.kr

*Contributed equally

Abbreviations: CDK, cell cycle division kinase

Key words: cyclin E, p21$^{WAF1/CIP1}$, mitomycin C, doxorubicin, hepatocellular carcinoma

Introduction

Most anticancer drugs disrupt DNA metabolism and induce DNA damage, and are thus cytotoxic against actively growing tumor cells. The potential molecular targets of such drugs have been intensively investigated with a particular focus on cell cycle and checkpoint control proteins. Cyclins, and the catalytic partners thereof, the cyclin-dependent kinases (CDKs), feature strongly in such work. Aberrant expression of such proteins, particularly cyclin D_1 and cyclin E, that control the G_1 phase of the cell cycle, is found in a variety of human cancers (1,2). Overexpression of cyclin D1 and cyclin E accelerates the G_1-S-phase transition (3), causes chromosomal instability (4), and alters the sensitivity of cells to anticancer agents (5). G_1 cyclin action is inhibited upon induction of p21$^{WAF1/CIP1}$, and occurs when an anticancer drug induces DNA damage (6).

Mitomycin C, an antibiotic produced by *Streptomyces caespitosus*, is a bioreductive anticancer drug, the reactive forms of which are created via several metabolic steps (7). Metabolically reactive mitomycin C induces DNA damage via DNA-alkylation, DNA-mono-adduct formation, and intra-strand cross-linking (8,9). Such reactions eventually result in antitumor effects in a broad range of human cancers (10-13). Doxorubicin is another useful wide-spectrum anticancer drug with a mechanism of action distinct from that of mitomycin C (14). Doxorubicin efficiently intercalates between DNA bases, and a ternary drug-DNA-topoisomerase II 'cleavable complex' (15,16) is subsequently formed. Such structural changes ultimately inactivate topoisomerase, and excision and rejoining

of the damaged region cannot proceed (17). Thus, although both drugs damage the DNA of cancer cells, mitomycin C and doxorubicin differ in mode of action. However, both mitomycin C- and doxorubicin-induced apoptosis is commonly linked to formation of reactive oxygen species created via redox activation of the drugs (18-21).

Both mitomycin C and doxorubicin are intra-arterially infused in efforts to increase survival of patients with unresectable advanced hepatocellular carcinoma (22,23). However, the side effects associated with the two drugs are severe and the response rate low; these constitute major obstacles to the use of such drugs as whole-body therapeutic regimens (24). There is therefore relatively little data on responses to such drugs by human hepatocellular carcinoma patients, compared with the information available on responses by patients with other cancers in whom the drugs exert useful effects. In the present study, we characterized the response to mitomycin C and doxorubicin of the human hepatocellular carcinoma cell lines HepG2 and Hep3B, which are of p53 status wild-type and null (25), respectively. Upon analysis of proteins functioning during the G_1 phase of the cell cycle, it became clear that the two anticancer drugs generated conflicting signals, inducing accumulation of cyclin E prior to that of p21$^{WAF1/CIP1}$ in both cell lines. The drug-induced accumulation of cyclin E was both rapid and irrespective of p53 status when the drugs were tested at lethal or sublethal concentrations. This constitutes the first indication that mitomycin C- and doxorubicin-induced accumulation of cyclin E is associated with the G_1-S-phase transition in human hepatocellular carcinoma cells.

Materials and methods

Cell culture and reagents. The human hepatocellular carcinoma cell lines HepG2 and Hep3B were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were maintained in minimum essential medium (MEM) (Gibco, Gaithersburg, MD, USA) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (Gibco), at 37˚C under an atmosphere of 95% air and 5% CO_2. The anticancer drugs used were doxorubicin (catalog no. D1515, Sigma, St. Louis, MI, USA) and mitomycin C (catalog no. 0503, Sigma). The antibodies employed were anti-cyclin E (catalog no. SC-198, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cyclin D_1 (catalog no. SC-718, Santa Cruz Biotechnology), anti-p21$^{WAF1/CIP1}$ (catalog no. SC-397, Santa Cruz Biotechnology), anti-p53 (catalog no. OP03, Millipore, Billerica, MA, USA), and anti-cdk2 (catalog no. SC-163, Santa Cruz Biotechnology).

Analysis of cytotoxicity, viability, and DNA fragmentation. The cytotoxicities of mitomycin C and doxorubicin were measured in triplicate using sulforhodamine B dye (catalog no. S2902, Sigma) staining (26). Asynchronously growing cells ($5x10^3$) were seeded into 96-well plates in 100 μl of medium, and, 24 h later, various concentrations of the drugs were added. Three days later, the cells were fixed in 50 μl amounts of cold 50% (v/v) trichloroacetic acid (TCA), washed 5 times with tap water, and next allowed to dry at room temperature. The TCA-fixed cells were stained with 0.1% (w/v) sulforhodamine B solution for 15 min, rinsed with 0.1% (v/v) acetic acid, and next incubated in 10 mM unbuffered Tris (pH 10.5) for 5 min. Cytotoxicity was determined by calculating the absorbance of drug-treated cells relative to that of untreated control cells. The viability of drug-treated cells was evaluated by staining with 0.4% (w/v) trypan blue. The extent of DNA fragmentation was measured using an ELISA kit assessing cell death (catalog no. 11920685001, Roche Diagnostics, GmbH, Germany) according to the manufacturer's instructions.

Western blot analysis. Whole-cell extracts were obtained as lysates in RIPA buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.02 mM phenylmethylsulfonylfluoride, 0.1 mM NaF, 0.01 mM Na_3VO_4, and 0.01 mg/ml aprotinin]. Aliquots of the resulting supernatants were separated by SDS-PAGE (12.5%, w/v), transferred to Protran nitrocellulose transfer membranes (Schleicher & Schuell, GmbH, Germany), and probed with specific antibodies. Blots were developed using a secondary antibody (Santa Cruz Biotechnology) and visualized by use of an enhanced chemiluminescence kit (catalog no. SC-2048, Santa Cruz Biotechnology).

Immunoprecipitation and kinase assay. Cyclin E-dependent kinase activity was measured using a specific antibody against cyclin E, as previously described (27). Briefly, cells were suspended in 500 μl of isotonic lysis buffer [0.2% (v/v) NP-40, 140 mM NaCl, 0.1 mM NaF, 0.01 mM Na_3VO_4, 0.01 mg/ml aprotinin, 0.02 mM phenylmethylsulfonylfluoride, and 20 mM Tris-HCl (pH 7.4)]. Whole-cell extracts were precleared using protein G-agarose (catalog no. 11243233001, Roche Diagnostics) and incubated with anti-cyclin E antibody. Immune complexes were collected by absorption onto protein A- or G-agarose beads, and washed with isotonic lysis buffer and kinase assay buffer (10 mM $MgCl_2$ in 50 mM HEPES, pH 7.4). The final pellets were resuspended in 25 μl amounts of kinase assay buffer containing 1 μg histone H1 (catalog no. 11004875001, Roche Diagnostics), 10 μM ATP, and 5 μCi [γ-^{32}P] ATP (3,000 Ci/nmol; NEN, Waltham, MA). The enzymatic reaction was allowed to proceed at 37oC for 15 min and the samples were next subjected to SDS-PAGE (12.5%, w/v). To quantitate kinase activity, proteins were transferred onto nitrocellulose membrane filters and the extent of histone H1 phosphorylation measured by autoradiography.

Cell cycle analysis. Cell cycle status was analyzed by staining of nuclei with propidium iodide and by measuring DNA content using flow cytometry (Becton-Dickinson, Mountain View, CA, USA). Cells harvested at various timepoints after drug exposure were adjusted to $1x10^6$ cells/ml and stained with 200 μl amounts of pre-cooled propidium iodide solution prior to incubation for 10 min in the dark on ice. The proportion of cells in each phase of the cell cycle was determined by measuring the intensity of nuclear DNA staining by flow cytometry. Chicken erythrocyte nuclei (catalog no. 349523, Becton-Dickinson) were used as a control.

Results

Mitomycin C and doxorubicin cause rapid accumulation of cyclin E in both p53-defective and -proficient cells. In response to DNA damage induced by anticancer agents, cells arrest at

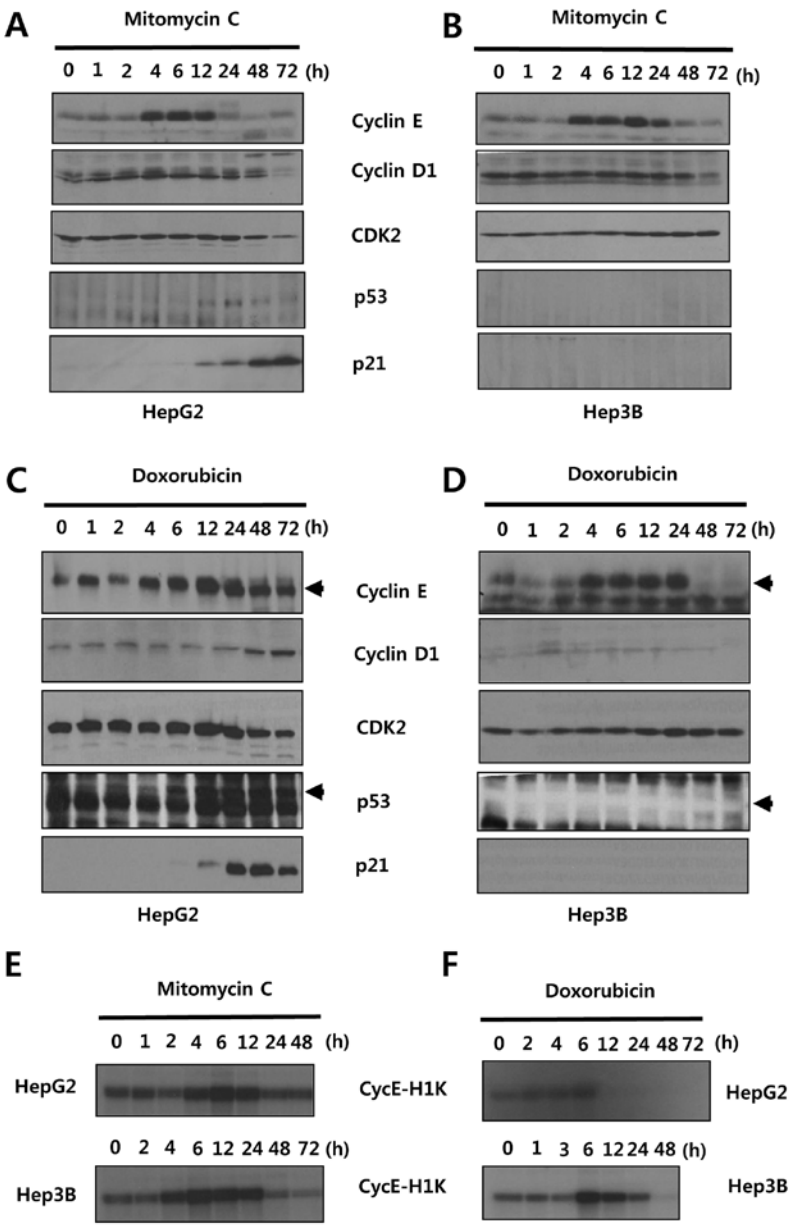

Figure 1. Rapid accumulation of cyclin E, prior to appearance of p53 and p21$^{WAF1/CIP1}$, after treatment with mitomycin C or doxorubicin. Either HepG2 (A and C) or Hep3B cells (B and D) were treated with 5 μM mitomycin C (A and B) or 1 μM doxorubicin (C and D). Whole-cell extracts were analyzed by sequential immunoblotting using antibodies against cyclin E, cyclin D_1, CDK2, p53, or p21$^{WAF1/CIP1}$. Cyclin E-associated protein kinase activity (CycE-H1K) was assayed in immune complexes precipitated using an anti-cyclin E monoclonal antibody (E and F).

a specific phase of the cell cycle and either repair the damage or enter the cell death pathway. To study the early response of damaged cells, in terms of both cell cycle arrest and death, we monitored expression of the cell cycle activators cyclin D1, cyclin E, and CDK2, and of the cell cycle inhibitor p21$^{WAF1/CIP1}$, all of which are active during the G_1 phase of the cycle (28). Initially, we focused on anticancer drug-induced cell death and estimated the concentrations of drugs affecting cell viability. Lethal concentrations of mitomycin C and doxorubicin, 5 μM and 1 μM, respectively, caused rapid accumulation of cyclin E in both HepG2 and Hep3B cells (Fig. 1). The accumulation of cyclin E was evident within 4 h after drug treatment and was sustained to 12-24 h. In terms of induction of cyclin E, the cyclin E-associated kinase was also activated in both HepG2 and Hep3B cells. As expected from consideration of p53 status, HepG2 cells produced p53 protein and the downstream target, p21$^{WAF1/CIP1}$ (Fig. 1A and C), but p53-null Hep3B cells did not (Fig. 1B and D). When the kinetics of cyclin E, p53, and p21$^{WAF1/CIP1}$ expression by HepG2 cells were analyzed, we found that the accumulation of cyclin E was more rapid than was that of p53 and p21$^{WAF1/CIP1}$ (Fig. 1A and C). DNA-damaging agents-induced p21$^{WAF1/CIP1}$ arrests the cell cycle; this has been well established using a variety of cells and organisms (29). In contrast to what was observed with cyclin E, cyclin D1 (which is also active in G_1 phase) and the CDK2 as catalytic partner, did not noticeably accumulate in response to genotoxic stress (Fig. 1A-D). Thus, cyclin E accumulation is an early response, occurring prior to increases in p53 and p21$^{WAF1/CIP1}$ levels after exposure to each mitomycin C and doxorubicin. Furthermore, cyclin E accumulation is not associated with p53 status. In

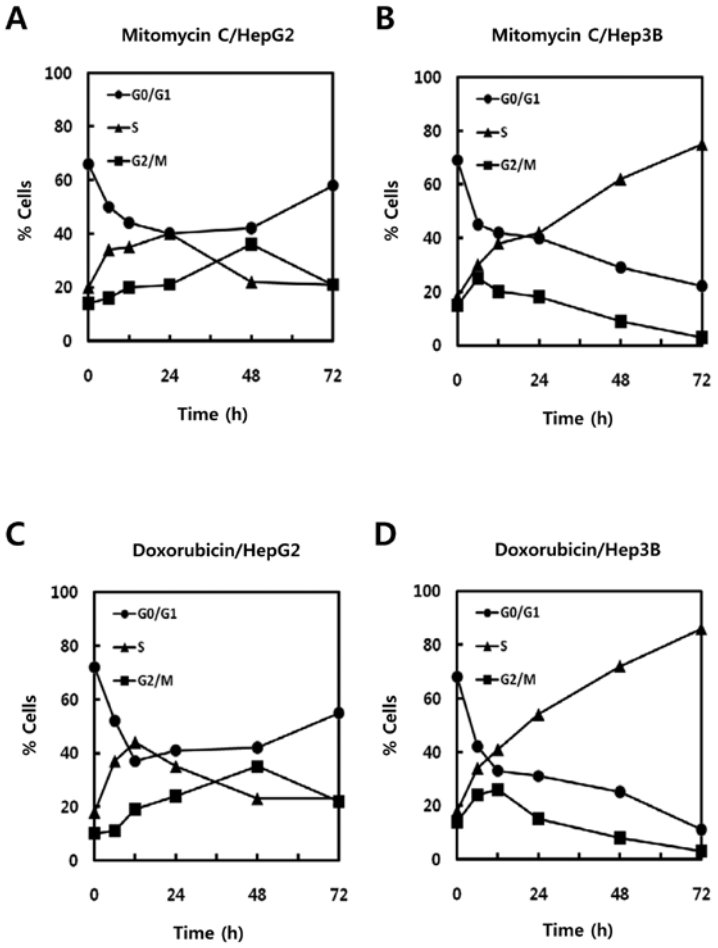

Figure 2. Changes in cell cycle phase distribution caused by mitomycin C and doxorubicin. Asynchronously growing HepG2 (A and C) and Hep3B (B and D) cells were treated with either 5 μM mitomycin C (A and B) or 1 μM doxorubicin (C and D) for the indicated times. Cell cycle distribution was next analyzed by flow cytometry after staining of nuclear DNA with propidium iodide, and the percentages of cells in the G_0/G_1, S, and G_2/M phases were determined using Becton-Dickinson software.

p53-proficient HepG2 cells, mitomycin C or doxorubicin elicit conflicting signals; both activation and inhibition of cell cycle progression are triggered, as indicated by induction of cyclin E and p21$^{WAF1/CIP1}$, respectively.

Mitomycin C- or doxorubicin-induced accumulation of cyclin E is associated with the G_1-S-phase transition. Since mitomycin C and doxorubicin induced accumulation of cyclin E in both HepG2 and Hep3B cells, and of p21$^{WAF1/CIP1}$ in HepG2 cells, we examined whether induction of these two molecules might affect cell cycle transition in response to genotoxic stress. Exposure of actively growing asynchronous cells to either drug resulted in a rapid increase of the proportion of cells in S-phase, accompanied by a concomitant loss of cells in G_1-phase, for up to 12 h after drug exposure (Fig. 2), indicating that the G_1-S-phase transition occurred early after exposure to such agents. Specifically, the cell population in S-phase increased from ~20% to ~40-50% upon exposure to mitomycin C (Fig. 2A and B) or doxorubicin (Fig. 2C and D). The increases in S-phase proportions were mainly attributable to decreases in G_1 population levels, as the 20-30% decrease in the G_1-phase population was proportional to that of the increase in S-phase cells. A minority of S-phase cells (~5-15%) moved into G_2/M phase during the same period. These results indicate that, upon exposure to mitomycin C or doxorubicin, cells in G_1 rapidly progress to S-phase.

When the kinetics of the G_1-S-phase transition were compared with those of cyclin E or p21$^{WAF1/CIP1}$ accumulation in HepG2 cells, it was evident that the phase transition was associated with accumulation of cyclin E rather than the increase in p21$^{WAF1/CIP1}$ level. The rapid transition from G_1-to S-phase occurred during the initial 12 h of drug exposure, when only cyclin E accumulation was evident (thus prior to distinct induction of p21$^{WAF1/CIP1}$) (Figs. 1A and C, and 2A and C). p21$^{WAF1/CIP1}$ was strongly induced 24-48 h after drug exposure (Fig. 1A and C). Such an association of the G_1-S-phase transition with cyclin E accumulation was also evident in Hep3B cells. In contrast to cessation of the G_1-S-phase transition evident 12 h after drug exposure in HepG2 cells, the transition continued in Hep3B cells (Fig. 2B and D), accompanied by cyclin E accumulation, up to 24 h post-treatment (Fig. 1B and D). In addition, the increase in the G_2/M population noted during the first 12 h ceased; G_2/M cells in fact decreased in number (Fig. 2B and D) even when cyclin E accumulation continued and p21$^{WAF1/CIP1}$ was absent (Fig. 1B and D). These results indicated that cyclin E accumulation was not associated with the S-G_2/M-phase transition. These findings indicate that, in response to mitomycin C and doxorubicin, cyclin E accumulation

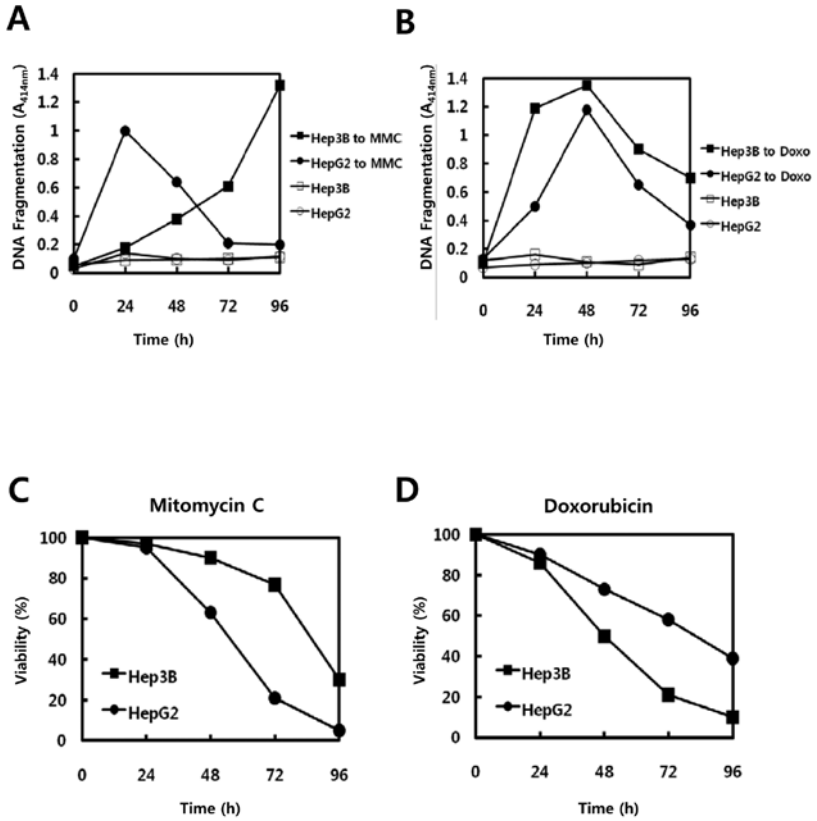

Figure 3. DNA fragmentation and cell viability after exposure to mitomycin C or doxorubicin. HepG2 and Hep3B cells were treated with either 5 μM mitomycin C (MMC) or 1 μM doxorubicin (Doxo) for the indicated times. (A and B) DNA fragmentation was assessed in HepG2 and Hep3B cells either exposed to (closed symbols) or not exposed to (open symbols) anticancer drugs, at the indicated time-points. (C and D) Loss of viability upon treatment with mitomycin C (C) or doxorubicin (D) was compared between HepG2 and Hep3B cells.

is associated with transition of the G_1-phase population to the S-phase in human hepatocellular carcinoma cell lines.

Mitomycin C- or doxorubicin-induced accumulation of cyclin E is not associated with DNA fragmentation or cell viability. Although we found a close correlation between cyclin E accumulation and G_1-S-phase transition in response to DNA-damaging agents, it was necessary to further investigate whether cyclin E accumulation was also associated with apoptotic cell death. It is known that the genotoxic stress-induced cyclin E fragment p18-cyclin E is associated with such death (30). Therefore, we compared the kinetics of cyclin E accumulation with those of DNA fragmentation and cell viability. Upon exposure to either mitomycin C or doxorubicin at 5 μM or 1 μM (the concentrations used in the experiments described above), DNA fragmentation was evident in both HepG2 and Hep3B cells (Fig. 3A and B). However, the kinetics of fragmentation were quite different (mitomycin C, Fig. 3A; doxorubicin, Fig. 3B), despite the fact that the kinetics of cyclin E accumulation were similar (Fig. 1). In response to mitomycin C, HepG2 cells rapidly (within 6 h) exhibited DNA fragmentation; this was maximal at 24 h (Fig. 3A). In contrast, Hep3B cells did not exhibit noticeable DNA fragmentation within 12 h (Fig. 3A), even though the cyclin E level was increasing (Fig. 1B). In addition, the extent of mitomycin C-induced DNA fragmentation was significant only 48 h after drug treatment (Fig. 3A), at which time cyclin E accumulation had ceased (Fig. 1B). These findings indicate that cyclin E accumulation was not associated with DNA fragmentation. The results obtained after exposure to doxorubicin further support the absence of an association between cyclin E accumulation and DNA fragmentation. In contrast to what was observed for mitomycin C, doxorubicin-induced DNA fragmentation in Hep3B cells was much faster than that in HepG2 cells (Fig. 3B). Mitomycin C- or doxorubicin-induced DNA fragmentation caused loss of viability of both HepG2 and Hep3B cells (Fig. 3C and D). Indeed, DNA fragmentation is typically seen in cells undergoing apoptosis (31). Thus, cyclin E accumulation is not associated with the DNA fragmentation or viability loss seen upon exposure of human hepatocellular carcinoma cell lines to mitomycin C or doxorubicin.

Mitomycin C- or doxorubicin-induced accumulation of cyclin E occurs at sublethal as well as lethal drug concentrations. Cell cycle arrest occurs during apoptotic cell death or cell growth cessation (32). Therefore, we examined the effects of sublethal concentrations of mitomycin C and doxorubicin on cell cycle arrest to determine whether cyclin E affected the G_1-S transition under such conditions. At mitomycin C levels of <1 μM, distinct loss of HepG2 cell viability was evident, although cell growth was inhibited in a concentration-dependent manner (Fig. 4); such drug concentrations were therefore sublethal in HepG2 and Hep3B cells. At such drug concentrations, mitomycin C caused rapid accumulation of cyclin E within 6 h in both cell lines, as was also evident at lethal concentrations (Fig. 5A and B). Consistently, an increase of the proportions of cells in S-phase

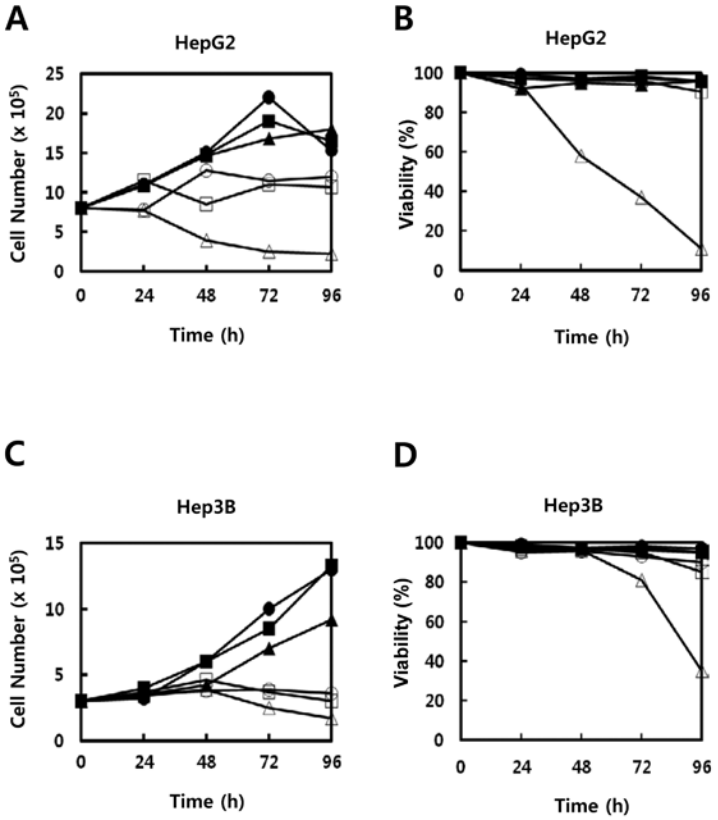

Figure 4. Comparison of cell growth retardation and viability loss upon exposure to mitomycin C. After treatment with mitomycin C, cell growth levels (A and C) and viabilities (B and D) of HepG2 and Hep3B cells were determined. The mitomycin C concentrations used were 0 μM (●), 0.008 μM (■), 0.04 μM (▲), 0.2 μM (○), 1 μM (□), and 5 μM (△).

was found within 24 h of exposure of HepG2 and Hep3B cells to sublethal drug concentrations of 0.2 and 1 μM, as well as to the lethal concentration used in the experiments described above (Figs. 5D, and 2A and B). Such enrichment of S-phase populations was also observed upon exposure of HepG2 cells to sublethal concentrations of doxorubicin, 0.04 and 0.2 μM (Figs. 6 and 7D), and was accompanied by cyclin E accumulation (Fig. 7A). Unlike the observed rapid cyclin E accumulation, p21$^{WAF1/CIP1}$ accumulation at sublethal drug concentrations was delayed, compared with that at lethal drug concentrations, or indeed disappeared, even when cyclin E was accumulating. Specifically, the mitomycin C-induced increase in p21$^{WAF1/CIP1}$ levels evident in HepG2 cells within 12 h of exposure to a lethal drug concentration of 5 μM was also seen 24 h and 48 h after exposure to sublethal drug concentrations of 1 and 0.2 μM, respectively (Fig. 5C). Also, sublethal concentrations of 0.2 and 0.4 μM doxorubicin led to the delayed appearance of p21$^{WAF1/CIP1}$ at 24 and 48 h post-treatment, respectively; accumulation after exposure to a lethal drug concentration of 1 μM was evident 12 h after treatment (Fig. 7C). Much lower sublethal concentrations that failed to induce p21$^{WAF1/CIP1}$ accumulation (Figs. 5C and 7C) could still cause cyclin E accumulation (Figs. 5A and 7A). Delayed accumulation of p21$^{WAF1/CIP1}$ allowed drug-exposed HepG2 cells to escape G_1-phase arrest, thus continuing the cell cycle transition, until eventually arresting in G_2/M (Figs. 5E and 7E). Thus, lethal drug concentrations caused cells to arrest at the G_0/G_1 or G_2/M phases, but sublethal concentrations of mitomycin C and doxorubicin arrested most cells at G_2/M (Figs. 5E, 7E, and F). Our present findings thus indicate that the G_1-S-phase transition induced by sublethal concentrations of anticancer drugs was not affected by p21$^{WAF1/CIP1}$ accumulation. Thus, cyclin E impacts the G_1-S-phase transition of hepatocellular carcinoma cell lines in the presence of sublethal or lethal concentrations of mitomycin C and doxorubicin.

Discussion

Mitomycin C and doxorubicin are used to treat a broad range of human cancers. Drug-DNA complexes are formed via cross-linking of drug with DNA or drug intercalation between DNA bases, thereby causing DNA damage and triggering cellular signaling cascades. To determine early signaling events in cells of cancers that respond poorly to the drugs *in vivo*, we monitored changes in the levels of proteins involved in G_1-phase arrest of human hepatocellular carcinoma cells. The response rates of such cells to anticancer drugs are low, and clinical applications are currently limited to intra-arterial chemotherapy.

In the present study, we showed that the two anticancer drugs rapidly induced accumulation of cyclin E (which functions in G_1 phase), but not cyclin D1, in both p53-expressing HepG2 and p53-null Hep3B cells. In HepG2 cells, accumulation of cyclin E was followed by p53-dependent accumulation of p21$^{WAF1/CIP1}$. Accumulation of cyclin E was required for the rapid transition of damaged cells from G_1- to S-phase. Subsequent accumulation of p21$^{WAF1/CIP1}$ determined the final state of cell cycle transition; this ceased in G_1 or G_2/M. Thus, conflicting signals, cyclin E and p21$^{WAF1/CIP1}$, induced by exposure to either mitomycin C or doxorubicin, acted coordinately to control cell cycle transition from

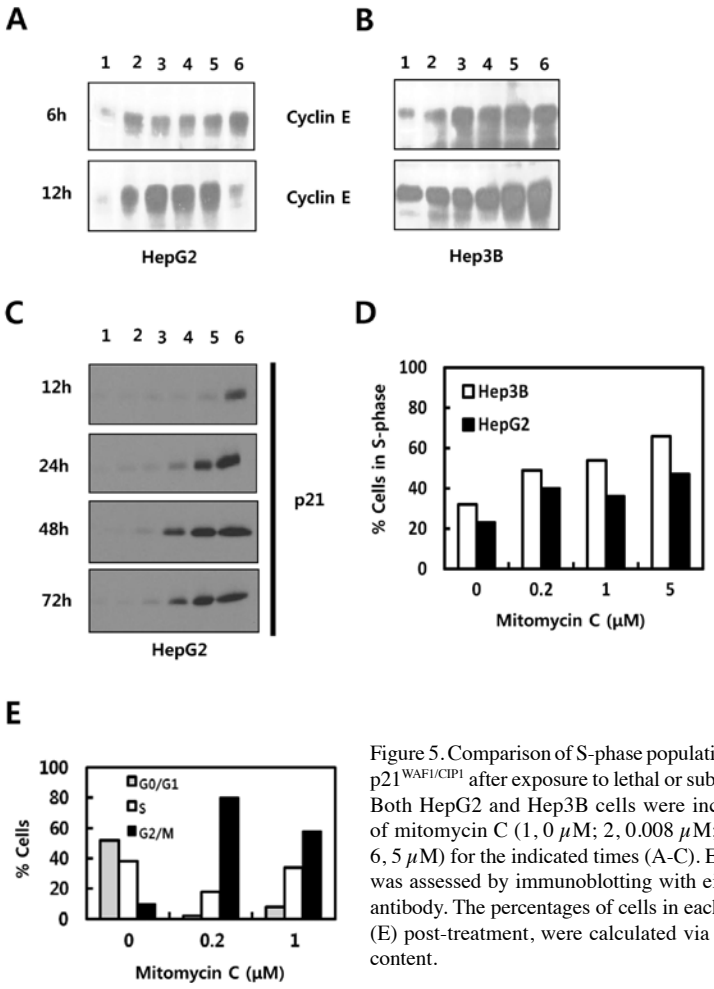

Figure 5. Comparison of S-phase population levels and expression of cyclin E or $p21^{WAF1/CIP1}$ after exposure to lethal or sublethal concentrations of mitomycin C. Both HepG2 and Hep3B cells were incubated with varying concentrations of mitomycin C (1, 0 μM; 2, 0.008 μM; 3, 0.04 μM; 4, 0.2 μM; 5, 1 μM; or 6, 5 μM) for the indicated times (A-C). Expression of cyclin E and $p21^{WAF1/CIP1}$ was assessed by immunoblotting with either anti-cyclin E or anti-$p21^{WAF1/CIP1}$ antibody. The percentages of cells in each phase of cell cycle, 24 h (D) or 72 h (E) post-treatment, were calculated via flow cytometric assessment of DNA content.

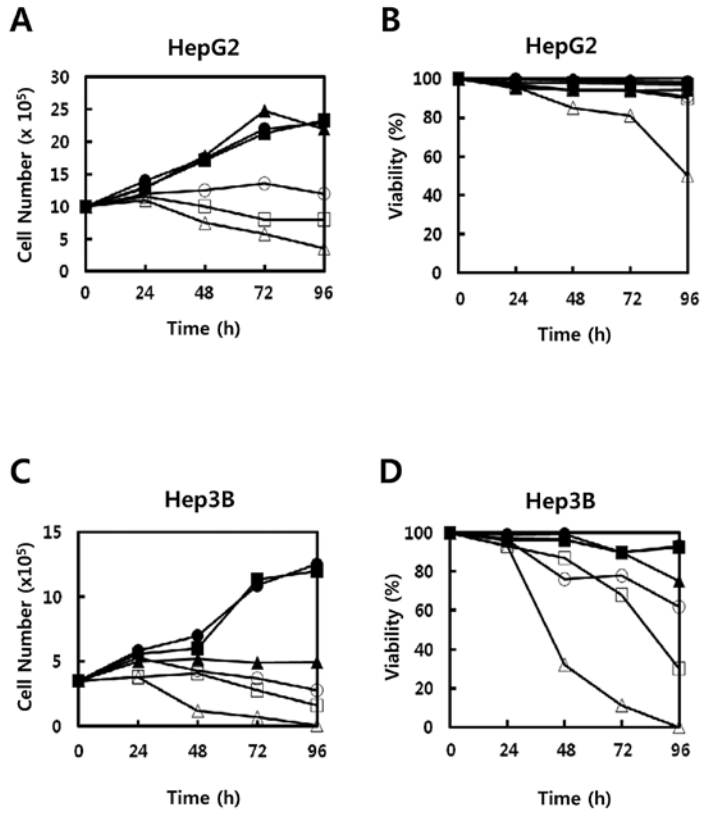

Figure 6. Comparison of cell growth retardation and viability loss upon exposure to doxorubicin. After exposure to doxorubicin, cell growth levels (A and C) and viabilities (B and D) of HepG2 and Hep3B cells were determined. The doxorubicin concentrations used were 0 μM (●), 0.0016 μM (■), 0.008 μM (▲), 0.04 μM (○), 0.2 μM (□), and 1 μM (△).

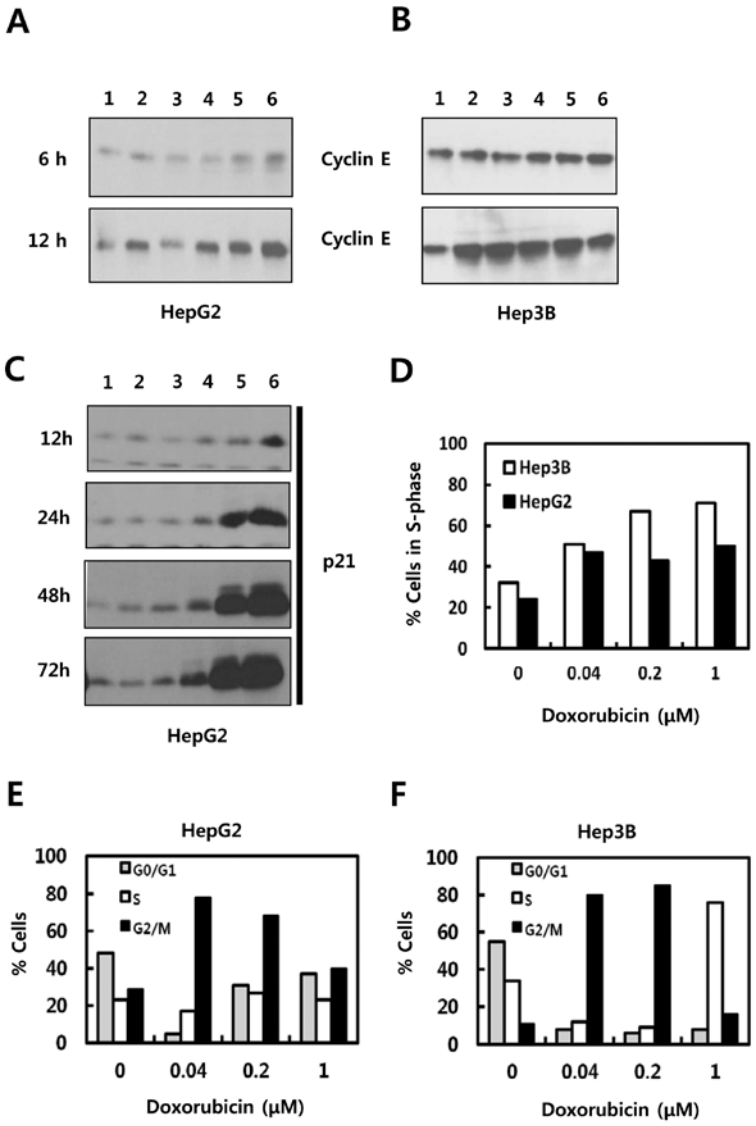

Figure 7. Comparison of S-phase population levels and expression of cyclin E or p21$^{WAF1/CIP1}$ after exposure to lethal and sublethal concentrations of doxorubicin. Both HepG2 and Hep3B cells were incubated with varying concentrations of doxorubicin (1, 0 μM; 2, 0.0016 μM; 3, 0.008 μM; 4, 0.04 μM; 5, 0.2 μM; or 6, 1.0 μM) for the indicated times (A-C). Expression of cyclin E and p21$^{WAF1/CIP1}$ was measured by immunoblotting with either anti-cyclin E or anti-p21$^{WAF1/CIP1}$ antibody. The percentages of cells in each phase of cell cycle, 24 h (D) or 72 h (HepG2, E and Hep3B, F), at 24 h or 72 h post-treatment, were calculated via flow cytometric assessment of DNA content.

G_1- to S-phase. In fact, cyclin E, but not cyclin D1, is aberrantly overexpressed in human hepatocellular carcinomas (33). Also, p21$^{WAF1/CIP1}$ is often not expressed in such cancers because *p53* mutations are often present (34). The overexpression of cyclin E and *p53* mutations often confer radio- and chemo-resistance of cancer cells (35,36). Thus, human hepatocellular carcinomas exhibiting both cyclin E overexpression and p53 mutations may not express the complicated and conflicting signals described above in response to such drugs. This may explain why the response rates of such carcinomas to anticancer drugs are low.

The anticancer drug-induced cell cycle transition from G_1- to S-phase, after attack by DNA-damaging anticancer drugs, differs somewhat from that seen upon growth stimulation of quiescent cells (this requires both cyclin D1 and cyclin E) (37,38). Indeed, ectopic overexpression of cyclin D1 or cyclin E shortens the retention time in G_1, thereby accelerating the G_1-S-phase transition (39). Therefore, cyclin E accumulation in response to an anticancer drug is the mechanism by which the progress of damaged cells through the cell cycle, from G_1- to S-phase, is controlled. Although cyclin D1 and cyclin E play similar roles in the cell cycle, in the sense that ectopic overexpression of either protein accelerates G_1-S-phase transition, several subtle between-protein differences are apparent. In particular, cyclin D1 overexpression causes the immediate synthesis of hyperphosphorylated pRb; this is not the case when cyclin E is overexpressed (39). Notably, the effect of cyclin E in asynchronous culture is greater than that of cyclin D1, whereas cyclin D1 has a greater impact when cells are stimulated from quiescence to growth (39). Addition of growth-competence agents, such as serum growth factors, to quiescent cells evoke cell cycling via cyclin D1 synthesis followed by that of cyclin E. However, our present results showed that both mitomycin C- and doxorubicin-induced cell cycle acceleration proceeded via accumulation of cyclin E but not cyclin D1. Thus, upon addition of either mitomycin or doxorubicin to human

hepatocellular carcinoma cells, cyclin E accumulation triggered rapid cell cycle transition.

Although we showed that anticancer drug-induced accumulation of cyclin E was clearly associated with the G_1-S-phase transition, we could not exclude the possibility that cyclin E accumulation was also linked to apoptosis. Indeed, genotoxic stress induces both cyclin E and an associated CDK2 activity (40), and cyclin E induction is linked to apoptosis (41). However, the fact that sublethal concentrations of mitomycin C and doxorubicin also caused accumulation of cyclin E eliminated the possibility of an association between such accumulation and apoptosis. Furthermore, the onset of cyclin E accumulation and DNA fragmentation differed when the two drugs were tested in the same cell line and between cell lines exposed to the same drug. Thus, mitomycin C- or doxorubicin-induced cyclin E accumulation is associated with the G_1-S-phase transition. However, the 18-kDa fragment of cyclin E, p18-cyclin E, plays a role in apoptosis via interaction with Ku70 (42). p18-cyclin E is derived by proteolytic cleavage of full-length cyclin E during apoptosis. This form of cyclin E cannot bind to the catalytic subunit CDK2, and is thus not involved in cell cycle progression (30). Therefore, genotoxic stress-induced full-length cyclin E and p18-cyclin E differ in terms of biological activity; full-length cyclin E affects the G_1-S-phase transition, but p18-cyclin E influences apoptosis.

Upon exposure to drugs of p53-proficient HepG2 cells, $p21^{WAF1/CIP1}$ accumulation, following the increase in cyclin E levels, ultimately caused cell cycle arrest. Unlike what was noted when cyclin E accumulation occurred rapidly, sublethal levels of mitomycin C and doxorubicin (irrespective of concentration) delayed $p21^{WAF1/CIP1}$ accumulation, permitting the cell cycle to continue; most cells ultimately arrested in G_2/M. In fact, $p21^{WAF1/CIP1}$ has a role in the G_1 checkpoint of the cell cycle (43). A deficiency of $p21^{WAF1/CIP1}$ causes loss of the G_1 checkpoint (44). Thus, HepG2 cells expressing $p21^{WAF1/CIP1}$ arrest at either G_1 or G_2/M depending on the concentration of anticancer drug present.

In our present work, we precisely dissected the kinetics of accumulation of both a cell cycle accelerator, cyclin E, and a cell cycle-inhibitory protein, $p21^{WAF1/CIP1}$. The data indicate that mitomycin C- and doxorubicin-induced cyclin E accumulation is coordinated with $p21^{WAF1/CIP1}$ expression to control the G_1-S-phase transition in human hepatocellular carcinoma cells. However, it is necessary to further investigate why cancer cells, including those of hepatocellular carcinomas, generate conflicting signals, both accelerating and inhibiting progression of the cell cycle.

Acknowledgments

This work was supported by a grant from the National Nuclear R&D program of the Korean Ministry of Education, Science and Technology. M.H.C. was supported by a grant from the National Research Foundation (no. NRF-2011-0000380) of the Korean Ministry of Education, Science and Technology.

References

1. Shapiro GI: Cyclin-dependent kinase pathways as targets for cancer treatment. J Clin Oncol 24: 1770-1783, 2006.
2. Kosacka M, Piesiak P, Porebska I, Korzeniewska A and Jankowska R: [The cyclin A, B1, D1 and E expression in advanced non-small cell lung cancer--stages IIIB-IV (preliminary report)]. Pol Merkur Lekarski 30: 253-258, 2011.
3. Resnitzky D, Gossen M, Bujard H and Reed SI: Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. Mol Cell Biol 14: 1669-1679, 1994.
4. Hubalek MM, Widschwendter A, Erdel M, Gschwendtner A, Fiegl HM, Müller HM, Goebel G, Mueller-Holzner E, Marth C, Spruck CH, Reed SI and Widschwendter M: Cyclin E dysregulation and chromosomal instability in endometrial cancer. Oncogene 23: 4187-4192, 2004.
5. Chung H, Chaudhry J, Lopez CG and Carethers JM: Cyclin E and histone H3 levels are regulated by 5-fluorouracil in a DNA mismatch repair-dependent manner. Cancer Biol Therb 10: 1147-1156, 2010.
6. He G, Kuang J, Huang Z, Koomen J, Kobayashi R, Khokhar AR and Siddik ZH: Upregulation of p27 and its inhibition of CDK2/cyclin E activity following DNA damage by a novel platinum agent are dependent on the expression of p21. Br J Cancer 95: 1514-1524, 2006.
7. Sartorelli AC, Hodnick WF, Belcourt MF, Tomasz M, Haffty B, Fischer JJ and Rockwell S: Mitomycin C: a prototype bioreductive agent. Oncol Res 6: 501-508, 1994.
8. Shinohara K, Bando T, Sasaki S, Sakakibara Y, Minoshima M and Sugiyama H: Antitumor activity of sequence-specific alkylating agents: pyrolle-imidazole CBI conjugates with indole linker. Cancer Sci 97: 219-225, 2006.
9. Weng MW, Zheng Y, Jasti VP, Champeil E, Tomasz M, Wang Y, Basu AK and Tang MS: Repair of mitomycin C mono- and interstrand cross-linked DNA adducts by UvrABC: a new model. Nucleic Acids Res 38: 6976-6984, 2010.
10. Bradner WT: Mitomycin C: a clinical update. Cancer Treat Rev 27: 35-50, 2001.
11. Kang SG, Chung H, Yoo YD, Lee JG, Choi YI and 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 22: 174-181, 2001.
12. Shiozawa M, Nishimura K, Nonaka T, Yoshii T, Nakayama N, Motohashi O, Takagi S, Nakayama Y and Akaike M: [Three cases of radiotherapy combined with S-1 and mitomycin C for anal canal squamous cell carcinomas]. Gan To Kagaku Ryoho 37: 2941-2943, 2010.
13. Vrdoljak E, Boban M, Omrcen T, Hrepic D, Fridl-Vidas V and Boskovic L: Combination of capecitabine and mitomycin C as first-line treatment in patients with metastatic breast cancer. Neoplasma 58: 172-178, 2011.
14. Carvalho C, Santos RX, Cardoso S, Correia S, Oliveira PJ, Santos MS and Moreira PI: Doxorubicin: the good, the bad and the ugly effect. Curr Med Chem 16: 3267-3285, 2009.
15. Larsen AK, Escargueil AE and Skladanowski A: Catalytic topoisomerase II inhibitors in cancer therapy. Pharmacol Ther 99: 167-181, 2003.
16. Stepankova J, Studenovsky M, Malina J, Kasparkova J, Liskova B, Novakova O, Ulbrich K and Brabec V: DNA interactions of 2-pyrrolinodoxorubicin, a distinctively more potent daunosamine-modified analogue of doxorubicin. Biochem Pharmacol (In press).
17. Wilstermann AM and Osheroff N: Stabilization of eukaryotic topoisomerase II-DNA cleavage complexes. Curr Top Med Chem 3: 321-338, 2003.
18. Shuhendler AJ, O'Brien PJ, Rauth AM and Wu XY: On the synergistic effect of doxorubicin and mitomycin C against breast cancer cells. Drug Metabol Drug Interact 22: 201-233, 2007.
19. Collier AC, Pritsos KL and Pritsos CA: TCDD as a biological response modifier for Mitomycin C: oxygen tension affects enzyme activation, reactive oxygen species and cell death. Life Sci 78: 1499-1507, 2006.
20. Chang WT, Li J, Huang HH, Liu H, Han M, Ramachandran S, Li CQ, Sharp WW, Hamann KJ, Yuan CS, Hoek TL and Shao ZH: Baicalein protects against doxorubicin-induced cardiotoxicity by attenuation of mitochondrial oxidant injury and JNK activation. J Cell Biochem doi: 10.1002/jcb.23201, 2011.
21. Kotamraju S, Konorev EA, Joseph J and Kalyanaraman B: Doxorubicin-induced apoptosis in endothelial cells and cardiomyocytes is ameliorated by nitrone spin traps and ebselen. Role of reactive oxygen and nitrogen species. J Biol Chem 275: 33585-33592, 2000.
22. Kumari R, Sharma A, Ajay AK and Bhat MK: Mitomycin C induces bystander killing in homogeneous and heterogeneous hepatoma cellular models. Mol Cancer 21: 8:87, 2009.
23. Gish RG, Abou-Alfa GK and Tong MJ: Clinical roundtable monograph. Integrating recent data in managing adverse events in the treatment of hepatocellular carcinoma. Clin Adv Hematol Oncol 8: 2p preceding 4-15, 2010.

24. Chang HM, Jung KH, Kim TY, Kim WS, Yang HK, Lee KU, Choe KJ, Heo DS, Bang YJ and Kim NK: A phase III randomized trial of 5-fluorouracil, doxorubicin, and mitomycin C versus 5-fluorouracil and mitomycin C versus 5-fluorouracil alone in curatively resected gastric cancer. Ann Oncol 13: 1779-1785, 2002.
25. Bressac B, Galvin KM, Liang TJ, Isselbacher KJ, Wands JR and Ozturk M: Abnormal structure and expression of p53 gene in human hepatocellular carcinoma. Proc Natl Acad Sci USA 87: 1973-1977, 1990.
26. Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR: New colorimetric cytotoxicity assay for anticancer-drug screening. J Natl Cancer Inst 82: 1107-1112, 1990.
27. Koff A, Giordano A, Desai D, Yamashita K, Harper JW, Elledge S, Nishimoto T, Morgan DO, Franza BR and Roberts JM: Formation and activation of a cyclin E-cdk2 complex during the G1 phase of the human cell cycle. Science 257: 1689-1694, 1992.
28. Sherr CJ: G1 phase progression: cycling on cue (review). Cell 79: 551-555, 1994.
29. Rai R, Peng G, Li K and Lin SY: DNA damage response: the players, the network and the role in tumor suppression. Cancer Genom Proteomics 4: 99-106, 2007.
30. Mazumder S, Gong B, Chen Q, Drazba JA, Buchsbaum JC and Almasan A: Proteolytic cleavage of cyclin E leads to inactivation of associated kinase activity and amplification of apoptosis in hematopoietic cells. Mol Cell Biol 22: 2398-2409, 2002.
31. Muppidi J, Porter M and Siegel RM: Measurement of apoptosis and other forms of cell death (review). Curr Protoc Immunol Chapter 3: Unit 3.17, 2004.
32. Alenzi FQ: Links between apoptosis, proliferation and the cell cycle. Br J Biomed Sci 61: 99-102, 2004.
33. Ohashi R, Gao C, Miyazaki M, Hamazaki K, Tsuji T, Inoue Y, Uemura T, Hirai R, Shimizu N and Namba M: Enhanced expression of cyclin E and cyclin A in human hepatocellular carcinomas. Anticancer Res 21B: 657-662, 2001.
34. Elbendary AA, Cirisano FD, Evans AC Jr, Davis PL, Iglehart JD, Marks JR and Berchuck A: Relationship between p21 expression and mutation of the p53 tumor suppressor gene in normal and malignant ovarian epithelial cells. Clin Cancer Res 2: 1571-1575, 1996.
35. Scaltriti M, Eichhorn PJ, Cortés J, Prudkin L, Aura C, Jiménez J, Chandarlapaty S, Serra V, Prat A, Ibrahim YH, Guzmán M, Gili M, Rodríguez O, Rodríguez S, Pérez J, Green SR, Mai S, Rosen N, Hudis C and Baselga J: Cyclin E amplification/overexpression is a mechanism of trastuzumab resistance in HER2^{+} breast cancer patients. Proc Natl Acad Sci USA 108: 3761-3766, 2011.
36. Raju U, Nakata E, Mason KA, Ang KK and Milas L: Flavopiridol, a cyclin-dependent kinase inhibitor, enhances radiosensitivity of ovarian carcinoma cells. Cancer Res 63: 3263-3267, 2003.
37. Resnitzky D and Reed SI: Different roles for cyclins D1 and E in regulation of the G1-to-S transition. Mol Cell Biol 15: 3463-3469, 1995.
38. Blagosklonny MV and Pardee AB: The restriction point of the cell cycle. Cell Cycle 1: 103-110, 2002.
39. Resnitzky D, Gossen M, Bujard H and Reed SI: Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. Mol Cell Biol 14: 1669-1679, 1994.
40. Mazumder S, Plesca D and Almasan A: A jekyll and hyde role of cyclin E in the genotoxic stress response: switching from cell cycle control to apoptosis regulation. Cell Cycle 6: 1437-1442, 2007.
41. Mazumder S, Gong B and Almasan A: Cyclin E induction by genotoxic stress leads to apoptosis of hematopoietic cells. Oncogene 19: 2828-2835, 2000.
42. Mazumder S, Plesca D, Kinter M and Almasan A: Interaction of a cyclin E fragment with Ku70 regulates Bax-mediated apoptosis. Mol Cell Biol 27: 3511-3520, 2007.
43. Lafarga V, Cuadrado A, Lopez de Silanes I, Bengoechea R, Fernandez-Capetillo O and Nebreda AR: p38 mitogen-activated protein kinase- and HuR-dependent stabilization of p21(Cip1) mRNA mediates the G(1)/S checkpoint. Mol Cell Biol 29: 4341-4351, 2009.
44. Lee J, Kim JA, Barbier V, Fotedar and Fotedar R: DNA damage triggers p21^{WAF1}-dependent Emi1 down-regulation that maintains G2 arrest. Mol Biol Cell 20: 1891-1902, 2009.