Replication-dependent γ-H2AX formation is involved in docetaxel-induced apoptosis in NSCLC A549 cells

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Abstract. Docetaxel is a member of the taxane anti-microtubule class of chemotherapeutic agents, which are currently widely used in clinical cancer therapy. However, the anti-tumor mechanisms of docetaxel are not fully understood. Herein we show that docetaxel induces dose-dependent apoptosis in non-small cell lung cancer A549 cells, as detected by Annexin-V positive cells and PARP cleavage, which is via mitochondrial pathway and dependent on caspase-3 activation. Our study on the mechanisms confirms that docetaxel induces dose-dependent accumulation of cells in M phase and acetylation of α-tubulin, marker of tubulin stablization. Furthermore, docetaxel induces replication-dependent γ-H2AX formation which plays a crucial role in docetaxel-triggered apoptosis. The DNA polymerase inhibitor aphidicolin dose-dependently prevents docetaxel-induced γ-H2AX formation, as well as apoptosis. Notably, 0.6 μM APC almost completely blocked docetaxel-induced γ-H2AX formation and apoptosis. In addition, wortmannin pretreatment caused elevated γ-H2AX level, which was accompanied with increased apoptosis. This effect was due to the inhibition of DNA repair process by wortmannin, as down regulation of $p21^{Waf1/Cip1}$ and DNA repair proteins such as Ku70, Ku80, DNA-PKcs and Rad50, were detected. These data show, for the first time, that the induction of apoptosis by docetaxel requires DNA replication, and replication-mediated DSBs are critical triggers of docetaxel-induced apoptosis.

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Abbreviations: DTX, docetaxel; NSCLC, non-small cell lung cancer; DSBs, DNA double-strand breaks; MMP, mitochondrial transmembrane potential; DNA-PK, DNA-dependent protein kinase

Key words: docetaxel, replication, γ-H2AX, apoptosis, non-small cell lung cancer

Introduction

Taxanes are natural products obtained from plants, which are currently widely used in clinical therapy of cancers. Taxanes bind to free tubulin subunits and promote assembly of stable microtubules and inhibit their disassembly, thereby blocking cell cycle progression in metaphase of mitosis, followed by induction of apoptosis and cell death (1,2). Two taxanes, paclitaxel and docetaxel have been used and shown great promise in the treatment of variety of cancers, including ovary (3), breast (4), melanoma (5) and lung (6) cancers.

The newer member of taxane class, docetaxel (DTX), is the semisynthetic analog of paclitaxel and is among the most potent cytotoxic drugs in clinical use. Although this agent has been studied extensively, the mechanisms of its anti-tumor action remain incompletely understood. Apart from the inhibition of microtubule dynamics, Cunha *et al* (7) also showed genotoxic effect of DTX. In addition, exposure of ovarian cancer cells to DTX led to p53 expression and induction of its downstream target $p21^{Waf1/Cip1}$ (8), suggesting that DNA damage might be induced in DTX-treated cells.

DNA double-strand breaks (DSBs) are the most dangerous type among DNA damage generated by ionizing radiation (IR) and other genotoxic agents. At sites of DNA DSBs, the histone H2AX is rapidly phosphorylated at serine 139 (phosphorylated H2AX, γ-H2AX). The formation of γ-H2AX causes chromatin decondensation and appears to play a critical role in the recruitment of repair or apoptotic-signaling factors to the sites of DNA damage. Apart from IR damage, which generates DSBs by itself, some anticancer agents have been demonstrated to induce replication-mediated DSBs (RepDSBs), which are apparently secondary lesions arising from primary DNA lesions, such as camptothecin (9), N2-G-Alkylator (10) and cisplatin (11). Ochs and Kaina (12) have reported that methylating agents such as O^6-methylguanine induce replication-dependent DSBs which are ultimate triggers of apoptosis. It has been concluded that DNA replication is a necessary component in DNA damage-triggered apoptosis in

fibroblasts by genotoxins not inducing DSBs themselves (13). This group further confirms that DSBs are critical ultimate apoptotic lesions specifically efficient in triggering apoptotic mitochondrial pathway (14).

Since DNA damage is the major cause of genotoxic agent-induced apoptosis, we wondered whether DNA damage is involved in DTX-induced apoptosis in non-small cell lung cancer (NSCLC) cells. In the current study, we show that replication-mediated DSBs are involved in DTX-triggered apoptosis in NSCLC A549 cells. Agent which inhibits DNA repair process and enhances DSBs level could increase DTX-induced apoptosis.

Materials and methods

Cell culture and treatments. A549 cells were obtained from the China Center for Type Culture Collection. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% fetal bovine serum, 10 mg/ml antibiotics (penicillin and streptomycin) and 2 mmol/l L-glutamine. Docetaxel was from JiangSu HengRui Medicine Co. Ltd. Aphidicolin was purchased from Sigma (St. Louis, USA) and was dissolved in dimethyl sulfoxide (DMSO, Sigma). Wortmannin (Sigma) was dissolved in DMSO to pre-treat the cells and proper amount of DMSO was used as vehicle control.

Cell cycle and BrdU incorporation analysis. Cells were collected by trypsinization at indicated time points. 5-bromo-2-deoxyuridine (BrdU, Sigma, 100 μM) was pulsed 1 h before harvesting. Samples were fixed in 70% ethanol at 4˚C overnight. FITC labeled anti-BrdU antibody (BD Pharmingen, USA) and propidium iodide (PI, Sigma) staining were performed. BrdU incorporation and DNA content were analyzed by flow cytometry with FACScan (Becton Dickinson, Mountain View, CA, USA) using the CellQuest program (Becton Dickinson). Data were analyzed by WinMDI software.

Cell cycle and M phase analysis. Cells were collected by trypsinization and were fixed in 70% ethanol at 4˚C overnight. Samples were incubated with antibody against phosphor-histone H3 (Ser10) (H3 P-S10) Santa Cruz, Biotechnology Inc.) for 1 h followed by incubation with FITC-labeled secondary antibody. Propidium iodide (PI, Sigma) staining was then performed. H3 P-S10 level and DNA content were analyzed by flow cytometry with FACScan (Becton Dickinson) using the CellQuest program (Becton Dickinson). Data were analyzed by WinMDI software.

Annexin-V-FITC and PI staining. Following different treatments, both adherent and non-adherent cells were harvested by trypsin digestion and washed twice with PBS. The experiments were performed using the Annexin-V-FITC apoptosis detection kit (BD Pharmingen) according to the manual. Briefly, cell pellets were re-suspended in 100 μl binding buffer (15) and stained with 5 μl Annexin-V-FITC and 5 μl propidium iodide (PI) staining solution in the dark at room temperature (RT) for 15 min. The cell samples were analyzed by flow cytometry on a FACScan station with CellQuest software using the FL1 and FL2 range for Annexin-V-FITC and PI, respectively.

Flow cytometry assessment of the changes in mitochondrial transmembrane potential (MMP). MMP was measured by flow cytometry with MitoTracker Red (Invitrogen) probe which is a mitochondrion-selective stain concentrated by active mitochondria. The probe accumulates in normal mitochondria and the reduction of MMP leads to the release of the stain. The probe was dissolved in DMSO and diluted in PBS before use. Cells were treated with MitoTracker for 45 min before trypsinization. Cells were then washed twice with PBS and analyzed by flow cytometry on a FACScan station with Cell Quest software using the FL2 for MitoTracker Red staining.

Analysis of active caspase-3 and γ-H2AX. Cells were collected by trypsin and the experiments were performed following the manual. Briefly, cells were fixed and permeabilized using the Cytofix/Cytoperm™ kit (BD Pharmingen) for 20 min at RT, pelleted and washed with Perm/Wash™ buffer (BD Pharmingen). Cells were then stained with FITC labeled anti-caspase-3 active form (BD Pharmingen) or anti-γ-H2AX (Upstate) (60 min) followed by FITC-labeled goat anti-mouse IgG for 60 min at RT in the dark. Samples were analyzed by flow cytometry on a FACScan station with CellQuest software using the FL1 for FITC labeled caspase-3 active form or γ-H2AX.

Western blotting. Cells were lysed as decribed previously (15). Equal amounts of protein (20 μg protein each lane) were separated by SDS-PAGE and then transferred to nitrocellulose membranes (Hybond C, Amersham, UK). The membrane was incubated with primary antibody at 4˚C overnight. Antibody against polyadenylribosyl polymerase (PARP) was from BD Pharmingen, antibodies against $p21^{Waf1/Cip1}$ and acetyl-α-tubulin were purchased from Santa Cruz, γ-H2AX (Ser139) antibody was from Upstate Biotechnology Inc., antibodies against Ku70, K80, DNA-PKcs and Rad50 were from BD Transduction Laboratories™, and ß-actin antibody was purchased from Sigma. The following steps were performed as described previously (15).

Statistical analysis. Data are shown as means plus SD from 3 independent experiments. Statistical comparisons were made using Students' t-test. $P<0.05$ was considered to represent a statistically significant difference.

Results

Docetaxel induces M-phase arrest in NSCLC A549 cells. Taxanes are antimicrotubular agents which block the cells in the G2/M phase of the cell cycle. We first observed the effect of DTX on cell cycle changes. Our results showed that DTX induced dose-dependent G2/M phase arrest by BrdU incorporation and PI staining (Fig. 1A and B). Using anti-phospho-Histone H3 (Ser10), a mitosis marker, we further confirmed that this G2/M arrest was due to increased accumulation of cells in M phase (Fig. 1C). The initial effective dose of DTX was 5 nM with ~37% cells accumulated in M phase. When 20 nM DTX was used, over 70% cells were arrested in M phase.

Acetylation of α-tubulin is thought to be an indicator of tubulin stability (16), we observed acetylation status of

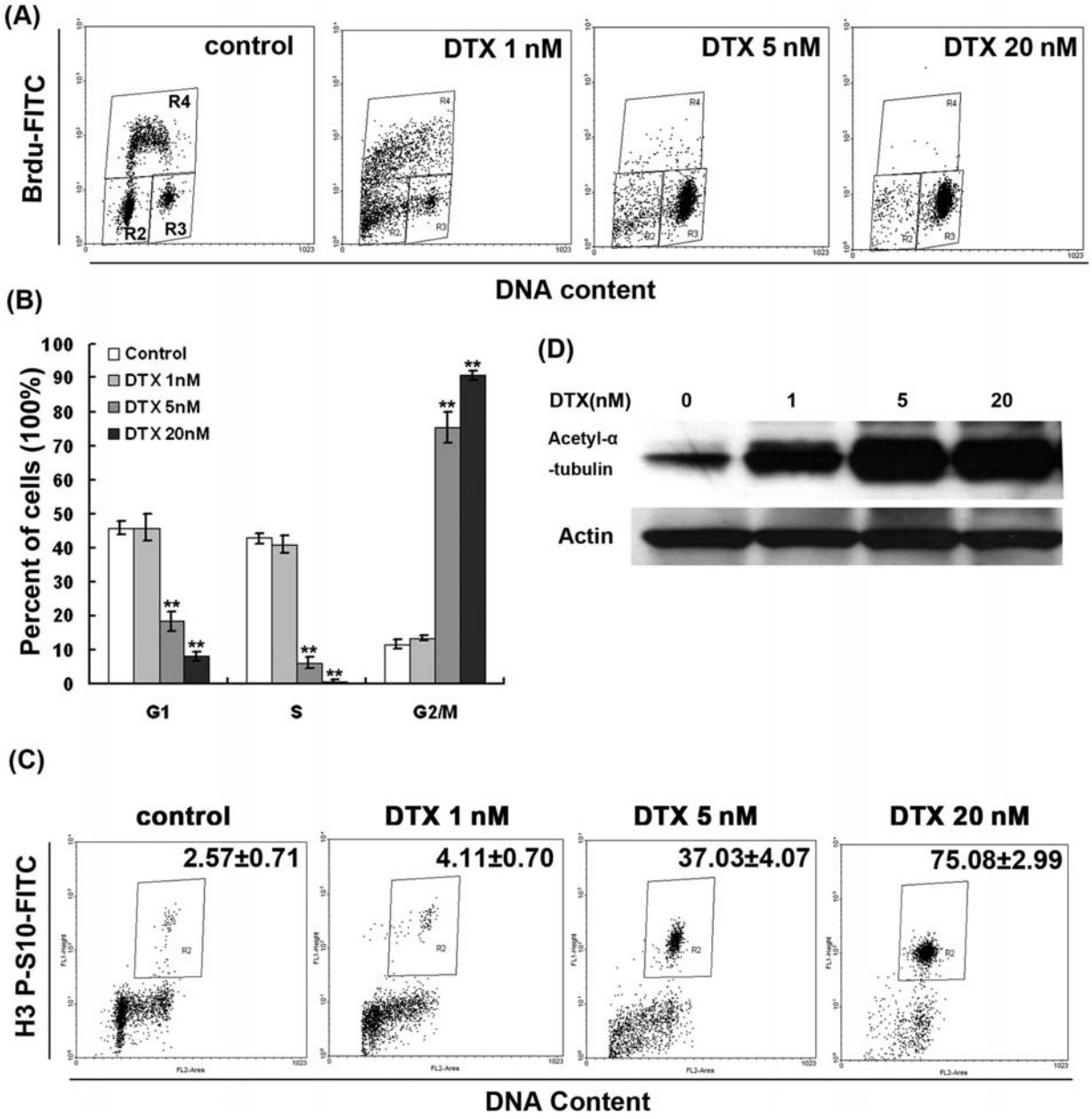

Figure 1. Docetaxel induces M-phase arrest in NSCLC A549 cells. A549 cells were exposed to different concentrations of docetaxel (DTX) (1, 5 and 20 nM) for 24 h. (A) Cells were collected for analysis of DNA content and BrdU incorporation by flow cytometry. Representative plots of one set of triplicate experiments are shown. In the first plot, region 2 (R2) represents G1 phase cells, region 3 (R3) G2 phase cells, and region 4 (R4) S phase cells. (B) Cell cycle distribution was analyzed and data are presented as means plus standard deviations (SD) (**P<0.01 compared to control). (C) Percentage of cells accumulated in M phase was analyzed using anti-phospho-Histone H3 Ser10 (H3 P-S10) and measured by flow cytometry. Data are presented as means plus SD. (D) Western blotting was performed using anti-acetyl-α-tubulin antibody, actin blot was included as loading control.

α-tubulin by Western blotting after DTX treatments. The results showed that DTX caused increased acetylation of α-tubulin in a dose-dependent manner (Fig. 1D), which is consistent with its effect of M phase arrest.

Docetaxel induces apoptosis in a dose-dependent fashion in A549 cells. We next measured DTX-induced apoptosis with Annexin-V-PI staining. A549 cells were exposed to varying concentrations of DTX (1, 5 and 20 nM) for 24 h. As shown in Fig. 2A and B, DTX caused dose-dependent apoptosis in A549 cells, and the initial effective dose of DTX was 1 nM. Apoptosis was further confirmed by PARP cleavage detected with Western blotting (Fig. 2C). The cleaved PARP 85 kD band was constantly detected in DTX treated cells in a similar pattern with Annexin-V positive cells.

Docetaxel induces apoptosis via mitochondrial pathway by causing dissipation of MMP and caspase-3 activation. It has been reported that caspase-3 activation and mitochondrial pathway are involved in DTX-induced apoptosis in human oral squamous carcinoma cells (17). We thus measured MMP dissipation, which plays a pivotal role in the initiation of apoptosis. The results showed that DTX caused increased loss of MMP in a dose-dependent manner (Fig. 3A). We next detected caspase-3 activation by flow cytometry with antibody against the active form of caspase-3. Our results showed that

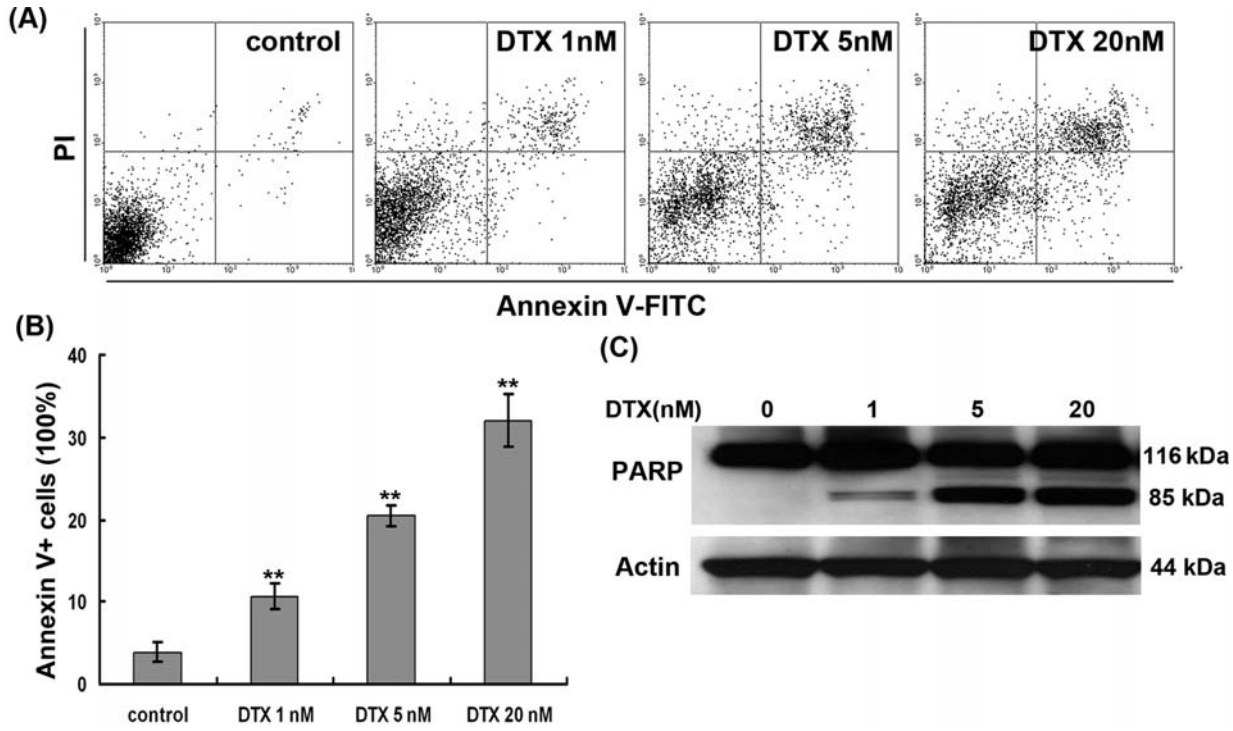

Figure 2. Docetaxel induces dose-dependent apoptosis in A549 cells. A549 cells were treated as described in Fig. 1. (A) Apoptosis was analyzed by Annexin-V-FITC and PI staining with flow cytometry. Representative plots of one set of triplicate experiments are shown. Early apoptotic cells (Annexin-V^+ and PI^-) are displayed in the lower right quadrant and late apoptotic cells (Annexin-V^+ and PI^+) are shown in the upper right quadrant. (B) The percentages of apoptotic cells are indicated by Annexin-V^+ cells shown as means plus SD from 3 independent experiments ($^{**}P<0.01$ compared to control). (C) Western blotting was performed using anti-PARP antibody, actin blot was included as loading control.

DTX induced caspase-3 activation in a similar manner with MMP dissipation (Fig. 3B). Caspase-3 inhibitor z-DEVD-fmk abolished DTX-induced caspase-3 activation, MMP dissipation, as well as apoptosis (Fig. 3C). Together, these results demonstrate that DTX-induced apoptosis in A549 cells is via mitochondrial pathway and dependent on caspase-3 activation.

Docetaxel induces replication-dependent γ-H2AX formation and apoptosis. Phosphorylation of histone H2AX on serine 139 generates phosphorylated H2AX (γ-H2AX), which is a sensitive and early marker for DNA DSBs. To investigate whether DTX induces DNA double-strand breaks (DSBs), we first detected γ-H2AX formation 24 h after drug treatment. The results (Fig. 4A) showed that DTX induced dose-dependent γ-H2AX formation as detected by flow cytometry.

To see whether the formation of DSBs by DTX depends on DNA replication, aphidicolin was used to pretreat cells, it inhibits DNA replication by inhibiting the binding of 2'-deoxynucleotides-5'-triphosphates (dNTPs) to DNA polymerases. The results from flow cytometry showed that aphidicolin markedly reduced the level of γ-H2AX in DTX-treated cells in a dose-dependent manner (Fig. 4B). These results were confirmed by Western blot analysis (Fig. 4C) using antibody against γ-H2AX. Furthermore, the prevention of γ-H2AX formation was accompanied with a decrease of apoptosis as measured by Annexin-V staining (Fig. 4B) and PARP cleavage (Fig. 4C). Taken together, these results strongly suggest that replication-dependent γ-H2AX formation plays an important role in DTX-induced apoptosis.

Wortmannin increases γ-H2AX level by inhibiting DNA repair, leading to enhanced apoptosis. To further demonstrate the importance of DNA damage in DTX-induced apoptosis, we used wortmannin to pretreat the cells. Wortmannin, a fungal metabolite, has been shown to have potent inhibitory effect on DNA DSBs repair, and has been proved to enhance the sensitivity of cancer cells to chemo- or radiotherapy. Herein, we showed that wortmannin enhanced DTX-induced apoptosis in a dose-dependent manner, while wortmannin alone (5 μM) had no effect on apoptosis compared to vehicle control (Fig. 5A and B). We then demonstrated that this effect was due to inhibition of DSBs repair as increased γ-H2AX formation with wortmannin pretreatment was observed by both flow cytometry and Western blotting (Fig. 5A and B).

$p21^{Waf1/Cip1}$ has been implicated in cell cycle arrest and DNA repair after DNA damage. $p21^{Waf1/Cip1}$ expression is activated upon DNA damage which causes cell cycle arrest in G1 or G2/M phase, allowing time for DNA repair. We also observed an increase of $p21^{Waf1/Cip1}$ expression after DTX treatment (Fig. 5C), which is consistent with a previous study by another group (8). However, wortmannin pretreatment significantly decreased DTX-induced $p21^{Waf1/Cip1}$ expression in a dose-dependent manner (Fig. 5D), suggesting that wortmannin may exert its inhibitory effect on DNA damage repair partly by downregulation of $p21^{Waf1/Cip1}$.

Ku proteins, including Ku70 and Ku80, are subunits of DNA-dependent protein kinase (DNA-PK) complex that are required for the DNA-binding activity of the complex on DNA breakage to facilitate DNA repair process (18). Rad50 also participates in the repair of DSBs by forming Mre11/Rad50/Nbs1 (MRN) complex which is a critical component in both homologous recombination and non-homologous end joining

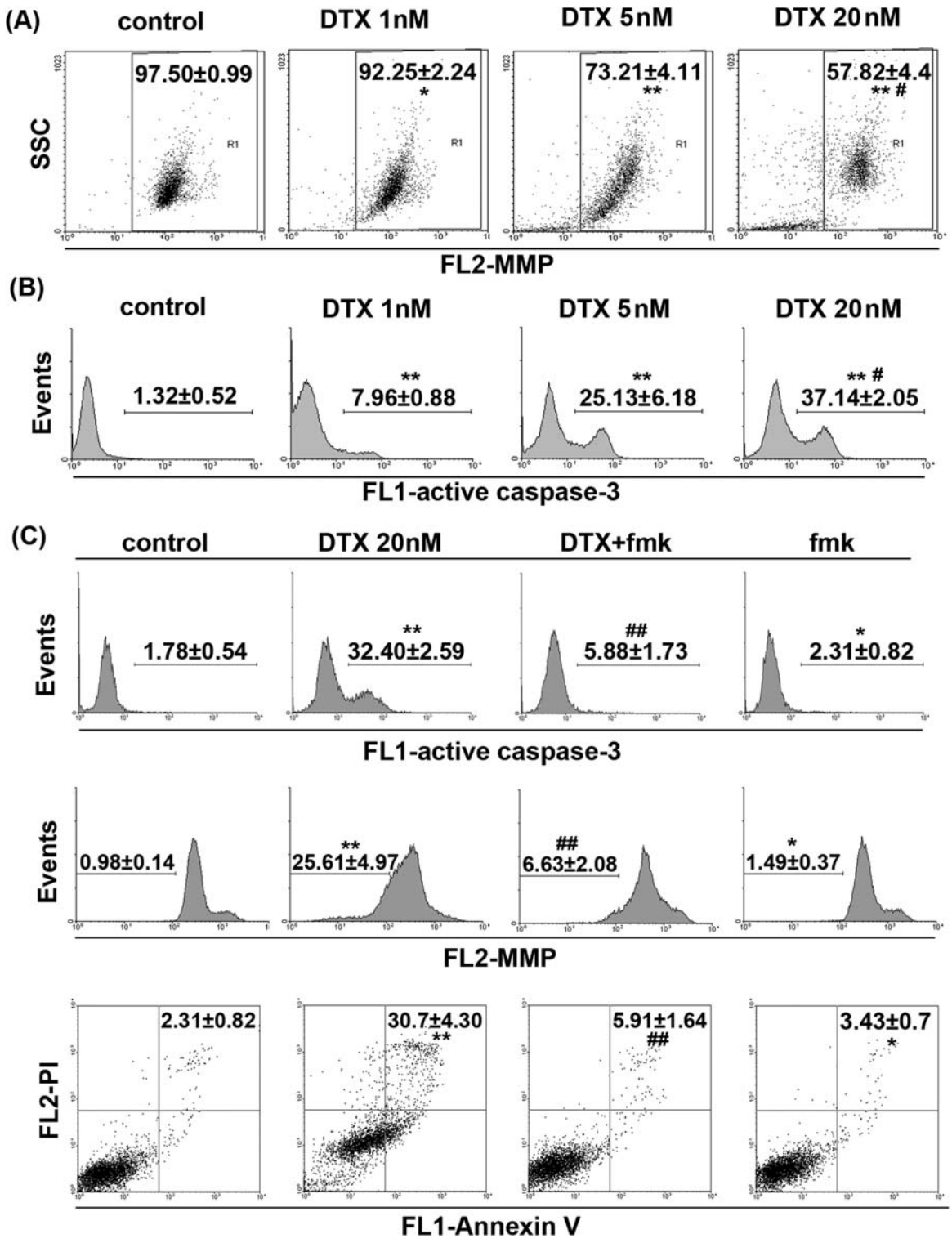

Figure 3. Docetaxel induces apoptosis via mitochondrial pathway by causing dissipation of MMP and caspase-3 activation. A549 cells were exposed to different concentrations of DTX (1, 5 and 20 nM) for 24 h. MMP changes (A) and active caspase-3 level (B) were measured by flow cytometry. Data are shown as means plus SD from 3 independent experiments with representative plots (*P<0.05, **P<0.01 compared to control and $^{\#}$P<0.05 compared to DTX 5 nM). (C) A549 cells were treated with DTX 5 nM, caspase-3 inhibitor z-DEVD-fmk (fmk) 100 μM or both for 24 h. Active caspase-3 level, MMP changes and Annexin-V positive cells were measured by flow cytometry. Data are shown as means plus SD from 3 independent experiments with representative plots (*P>0.05, **P<0.01 compared to control and $^{\#\#}$P<0.01 compared to DTX 20 nM).

pathways (19,20). We examined the effects of wortmannin treatment alone or in combination with docetaxel by Western blotting, on the expression of Ku70, Ku80, DNA-PKcs and Rad50. Our results showed that pretreatment with wortmannin decreased the expression of Ku70, Ku80, DNA-PKcs and Rad50.

Collectively, these results demonstrate that, by impairing DNA repair process, wortmannin enhances DTX-induced

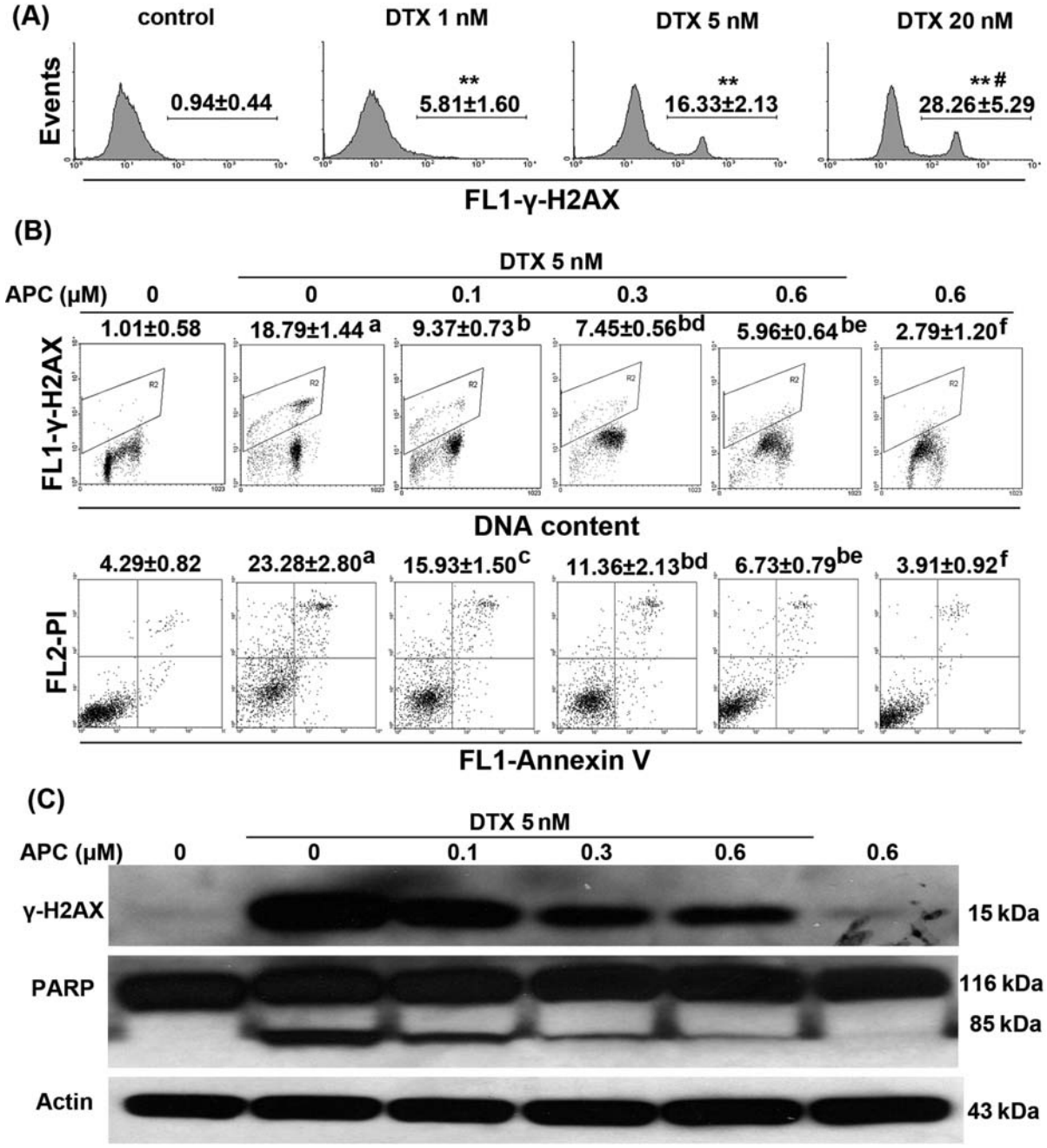

Figure 4. Docetaxel induces replication-dependent γ-H2AX formation and apoptosis. (A) A549 cells were exposed to different concentrations of DTX (1, 5 and 20 nM) for 24 h. γ-H2AX level was measured by flow cytometry. Data are shown as means plus SD from 3 independent experiments with representative plots (**P<0.01 compared to control and $^{\#}$P<0.05 compared to DTX 5 nM). (B) A549 cells were treated with DTX 5 nM, or DTX with aphidicolin (APC) 0.1, 0.3 or 0.6 μM, and APC 0.6 μM alone. γ-H2AX level and Annexin-V positive cells were measured by flow cytometry. Data are shown as means plus SD from 3 independent experiments with representative plots (^{a}P<0.01 compared to control, ^{b}P<0.01 and ^{c}P<0.05 compared to DTX group, ^{d}P<0.05 compared to APC 0.1 μM+DTX group, ^{e}P<0.05 compared to APC 0.3 μM+DTX group, and ^{f}P>0.05 compared to control). (C) Cell lysates from B were analyzed by Western blotting using antibodies against γ-H2AX and PARP with actin blot as loading control.

DSBs, which contributes to increased sensitivity to DTX-induced apoptosis in A549 cells.

Discussion

Non-small cell lung cancer (NSCLC) is the leading cause of cancer mortality in the world. Most NSCLC patients respond poorly to conventional chemotherapy because of the emergence of resistance. Hence, there is an urgent need to develop novel treatment strategies to improve chemosensitivity.

Docetaxel (DTX) is a member of the taxane anti-microtubule class of chemotherapeutic agents, which plays an important role in cancer therapy. It has been approved for both first- and second-line treatment of advanced NSCLC (21,22). However, the precise mechanisms of DTX are not yet fully understood. Several studies indicate that antimicrotubule agents are potent promoters of apoptosis in cancer

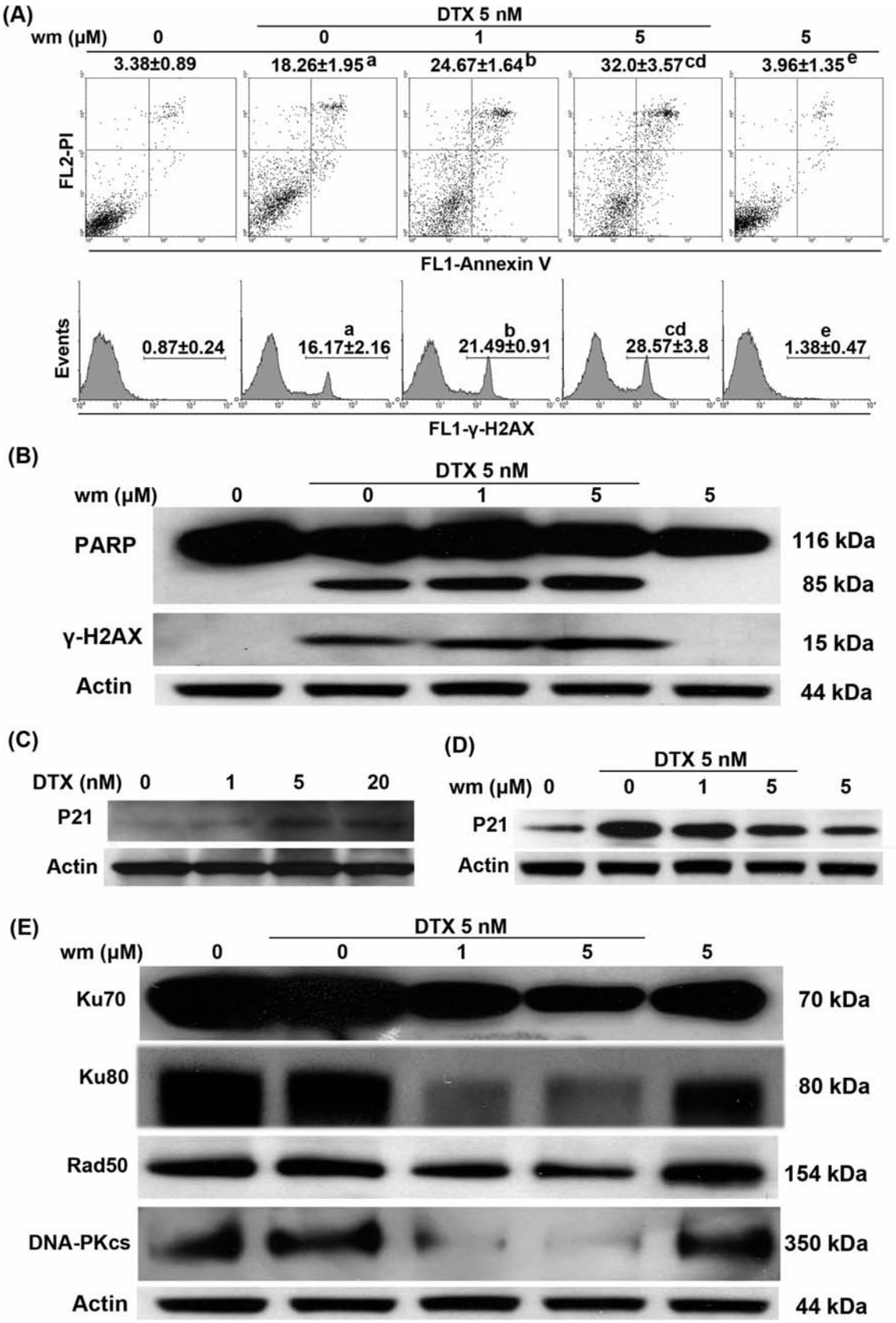

Figure 5. Wortmannin sensitizes A549 cells to docetaxel-induced apoptosis by repressing γ-H2AX level. (A) A549 cells were treated with DTX 5 nM, or DTX with wortmannin (wm) 1 or 5 μM, and wm 5 μM alone. Annexin-V positive cells and γ-H2AX level were measured by flow cytometry. Data are shown as means plus SD from 3 independent experiments with representative plots ([a]P<0.01 compared to control, [b]P<0.05 and [c]P<0.01 compared to DTX group, [d]P<0.05 compared to wm 1 μM+DTX group, [e]P>0.05 compared to control). (B) Cell lysates from A were analyzed by Western blotting using antibodies against PARP and γ-H2AX with actin blot as loading control. (C) A549 cells were exposed to different concentrations of DTX (1, 5 and 20 nM) for 24 h. Western blotting was performed using anti-$p21^{Waf1/Cip1}$ antibody with actin blot as loading control. (D) Cell lysates from A were prepared and analyzed by Western blotting using anti-$p21^{Waf1/Cip1}$ antibody with actin blot as loading control. (E) Cell lysates from A analyzed by Western blotting using antibodies against Ku70, Ku80, DNAPKcs and Rad50 with actin blot as loading control.

cells. In the current study, we demonstrate that DTX induces apoptosis in NSCLC A549 cells with increasing dissipation of MMP and activation of active caspase-3 in a dose-dependent manner. The caspase-3 inhibitor abolished DTX-induced loss of MMP and apoptosis, suggesting that mitochondrial pathway and caspase activation are involved in DTX-induced apoptosis.

Kolfschoten *et al* (8) have reported in four ovarian cancer cell lines that cells are first arrested in mitosis, followed by nuclear fragmentation, DNA degradation and subsequent apoptosis, proposing that G2/M arrest followed by apoptosis is a mechanism of DTX-induced cell death. We also confirm that DTX induces dose-dependent G2/M arrest which is due to M phase arrest. The results were further confirmed by the observations of α-tubulin acetylation in DTX-treated A549 cells, which is the marker for tubulin stabilization.

Previous studies have shown that the expression levels of p53 and its downstream target $p21^{Waf1/Cip1}$ increased in DTX-treated ovarian cancer cell lines expressing wild-type p53 (8), suggesting that DNA damage might be induced in DTX-treated cells. It is not clear whether DSBs play a role in apoptosis as a response to DTX. Phosphorylation of histone H2AX on serine 139 (γ-H2AX) is a sensitive and early marker for DSBs. We thus detected γ-H2AX formation 24 h after DTX treatment. Our results demonstrate that DTX induces dose-dependent γ-H2AX formation; furthermore, aphidicolin pretreatment prevents DTX-induced γ-H2AX formation, as well as apoptosis. From the above data, though the nature of the primary lesions generated by DTX are not clear, their processing can lead to formation of replication-associated DSBs, which ultimately trigger apoptosis. It could be concluded that the induction of apoptosis by DTX requires DNA replication.

Aphidicolin (23) is an inhibior of DNA polymerases which are required for DNA repair, as a result it could inhibit the DNA repair process. Aphidicolin has been proved to markedly increase the platinum sensitivity of cells from primary ovarian tumours (24). Using blast cells isolated from AML patients, Sargent *et al* (25) observed significant effect of aphidicolin on sensitivity to Ara-C [1-ß-D-arabino-furanosylcytosine (cytarabine)], while no significant increase in sensitivity to daunorubicin, doxorubicin, etoposide or fludarabine was observed. In the current study, we show that DTX induces replication-dependent DSBs and apoptosis, pretreatment with aphidicolin markedly inhibits sensitivity of A549 cells to DTX. These results suggest that the DNA repair inhibitor aphidicolin could modulate drug resistance in cancer cells, depending on the nature of the damage induced by different anticancer drugs. For agents which induce replication-dependent DSBs, aphidicolin combination prevents DSB formation and subsequent apoptosis.

Resistance to apoptosis is known to be a hallmark of various cancers, and therefore, combinational therapy has been suggested for clinical antitumor treatment. Since DTX plays an important role in NSCLC treatment, it is important to investigate the molecular mechanisms to provide evidence for choice of agents which could improve the sensitivity to DTX. We have investigated and reported several agents which have the capacity to increase the sensitivity of tumor cells to chemo- or radiotherapy, such as trichostatin A (15) and wortamnnin (26). One of the mechanisms of their effects is inhibition of DNA repair activity.

Upon DNA damage, $p21^{Waf1/Cip1}$ is activated and arrests cells in G1 or G2/M phase for DNA repair, which is one important reason for cancer cells to develop resistance. In the current study, we also observed an increase of $p21^{Waf1/Cip1}$ expression after DTX treatment. Interestingly, pretreatment with wortmannin caused a dose-dependent decrease of $p21^{Waf1/Cip1}$ expression, which was accompanied with a decrease of expression of DNA repair protein Ku70, Ku80, DNA-PKcs and Rad50, and an increase of sensitivity of A549 cells to DTX-induced apoptosis.

Furuta *et al* (9) have reported that the cell cycle checkpoint abrogator UCN-01 blocks $p21^{Waf1/Cip1}$ upregulation and induces γ-H2AX formation following camptothecin treatment. They also have demonstrated that replication mediated DNA DSBs induced by camptothecin and detected by γ-H2AX formation increase markedly in cells with a deficient p53/$p21^{Waf1/Cip1}$ pathway (27). We have reported that $p21^{Waf1/Cip1}$ cleavage may interfere with DNA repair, leading to increased frequency of DSBs and enhanced apoptosis by roscovitine (28). These observations imply an important role for $p21^{Waf1/Cip1}$ in the processing and repair of replication-mediated DSBs.

Wortmannin is a potent inhibitor of the catalytic subunit of DNA-PK which is involved in DNA DSB rejoining. Mirzayans *et al* (29) have shown that inhibition of DSB rejoining by wortmannin may be an important contributor to its radiosensitizing effect in A549 cells. Ortiz *et al* (30) have reported that the radiosensitizing effect of wortmannin on RT112 bladder tumor cells is a direct consequence of the inhibition of DNA-PK, resulting in the inhibition of DSB repair. Wortmannin also inhibits ATM, which is activated in response to DSBs and functions upstream in the p53/$p21^{Waf1/Cip1}$ signaling pathway. A recent study showed that wortmannin attenuated doxorubicin-induced ATM activation and p53 induction in cardiomyocytes (31). Thus, we may conclude that the increased levels of DTX-induced DSBs in wortmannin pretreated cells are due to the decrease of $p21^{Waf1/Cip1}$ induction and DNA repair activity. Lu *et al* (32) have reported that ultraviolet (UV) A irradiation induced H2AX phosphorylation that was mediated by c-Jun N-terminal kinase (JNK). Further study is needed to confirm the exact kinase mediating γ-H2AX formation upon docetaxel treatment.

In the current study, we have found that, apart from M phase arrest, DTX induces dose-dependent γ-H2AX foci and apoptosis in NSCLC A549 cells. Moreover, aphidicolin pretreatment prevents, while wortmannin pretreatment increases the formation of γ-H2AX in DTX-treated cells. Overall, the data indicate that the induction of apoptosis by DTX requires DNA replication, and replication-mediated DSBs are ultimate triggers of DTX-induced apoptosis. The current data have important implications for the use of DTX and the choice of other agents in combination with it. In addition, γ-H2AX may be used as a useful marker for monitoring the efficacy of DTX in clinical NSCLC treatment.

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