npg

Original Article

Induction of G$_2$/M arrest by pseudolaric acid B is mediated by activation of the ATM signaling pathway

Ai-guo MENG [1,2], Ling-ling JIANG [1,*]

[1]Department of Biochemistry, Hebei Medical University, Shijiazhuang 050017, China; [2]Affiliated Hospital, North China Coal Medical University, Tangshan 063000, China

Aim: The aim of this study was to investigate the mechanism of pseudolaric acid B (PLAB)-induced cell cycle arrest in human melanoma SK-28 cells.
Methods: Cell growth inhibition was detected by MTT assay, the cell cycle was analyzed by flow cytometry, and protein expression was examined by Western blot analysis.
Results: PLAB inhibited the growth of human melanoma cells and induced G$_2$/M arrest in SK-28 cells, accompanied by an up-regulation of Cdc2 phosphorylation and a subsequent down-regulation of Cdc2 expression. Furthermore, PLAB decreased the expression of Cdc25C phosphatase and increased the expression of Wee1 kinase. Meanwhile, a reduction in Cdc2 activity was partly due to induction of the expression of p21$^{waf1/cip1}$ in a p53-dependent manner. In addition, PLAB activated the checkpoint kinase, Chk2, and increased the expression of p53, two major targets of ATM kinase. These effects were inhibited by caffeine, an ATM kinase inhibitor. We also found that PLAB significantly enhanced ATM kinase activity.
Conclusion: Taken together, these results suggest that PLAB induced G$_2$/M arrest in human melanoma cells via a mechanism involving the activation of ATM, and the effect of PLAB on Cdc2 activity was mediated via interactions with the Chk2-Cdc25C and p53 signalling pathways, two distinct downstream pathways of ATM. PLAB may be a promising chemopreventive agent for treating human melanoma.

Introduction

Pseudolaric acid B (PLAB), a natural diterpenoid compound, is isolated from the root bark of *pseudolarix kaempferi Gordon*[1], a traditional Chinese medicine. It displays a wide spectrum of biological activities, including antifungal, anti-fertile, and anti-angiogenic properties[2–7]. Previous studies have shown that PLAB induces growth inhibition, cell cycle arrest and apoptosis in a variety of cancer cell lines, including breast cancer, colon cancer, hepatocellular carcinoma and melanoma cells[8–12].

The underlying molecular mechanisms of apoptosis induction and G$_2$/M cell cycle arrest induced by PLAB have been the focus of extensive research. PLAB was shown to induce apoptosis via activation of c-Jun N-terminal kinase

and caspase-3 in HeLa cells, through p53 and Bax/Bcl-2 pathways in human melanoma A375-S2 cells and through activation of JNK and inactivation of ERK in PLAB-treated human breast cancer MCF-7 cells[10–12]. A recent study reported that PLAB induced G$_2$/M arrest in AGS human gastric cancer cells via down-regulation of cdc2[13], but the molecular mechanism has not yet been elucidated.

Malignant melanoma is one of the most virulent forms of cancer, and advanced melanoma has a very poor prognosis. Therefore, the discovery of better anti-cancer agents against melanoma is an urgent goal. In contrast to many other aggressive and chemoresistant cancers, p53 mutations are rarely observed in melanoma. In this study, we evaluated the growth inhibition effect of PLAB on human melanoma cell lines including A372, SK-28 and 624 mel and further investigated PLAB's mechanism of action using SK-28 as a representative cell line model. In addition, we assayed the levels and phosphorylation status of proteins that are strongly associated with cell cycle progression. In particular, we highlight

the ability of PLAB to modulate Cdc2 activity by interacting with the Chk2-Cdc25C and p53 signaling pathways, two distinct downstream pathways of the related kinase, ataxia telangiectasia mutated kinase (ATM). We show that PLAB modulates Wee1 and p21$^{waf1/cip1}$ expression and Cdc25C phosphorylation status.

Materials and methods

Chemicals Dimethyl sulfoxide (DMSO), ribonuclease A (RNase A), propidium iodide (PI), Triton X-100, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazolium bromide (MTT), protein A-Sepharose, and other chemicals and cell culture medium RPMI-1640 were purchased from Sigma Chemical Co(USA). Horseradish peroxidase-conjugated secondary antibodies and antibodies against p53, p53-p, p21$^{waf1/cip1}$, Chk2, Cdc25C, Cdc25C-P, Cdc2, Cdc2-P, Cycling B1 and β-actin were purchased from Santa Cruz (CA, USA). Anti-ATM antibody was purchased from Oncogene Research Products. PLAB (C$_{23}$H$_{28}$O$_8$, FW432, Figure 1) was obtained by isolation from the raw herb by the Chinese Academy of Medical Science. All reagents were dissolved in DMSO at 10 mmol/L as a stock solution. The final concentration of DMSO in the culture medium did not exceed 0.1%. All stock solutions were stored at -70°C and were further diluted to appropriate concentrations with medium before use.
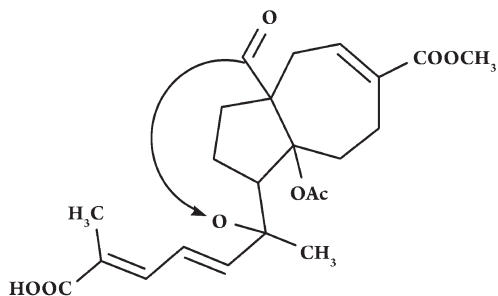

Figure 1. Chemical structure of pseudolaric acid B (PLAB).

Cell culture The human melanoma cell lines, A372, SK-28 and 624 mel, were obtained from the Cancer Research Center, Peking University. All cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin G and 100 μg/mL streptomycin (North China Pharmaceutical Group Corporation, China).

Cell proliferation assay The effect of PLAB on cell proliferation was measured by a modified MTT assay. A372, SK-28 and 624 mel cells (1.5×10^4 cells/well) were seeded on 96-well cell culture plates in a volume of 100 μL. On the following day, experimental medium containing different PLAB concentrations (0.01, 0.1, 1.0, and 10 μmol/L) was added and incubated for various times. MTT solution was added to each well and incubated for 4 h. After careful removal of the medium, 150 μL of DMSO was added to each well; after careful shaking, the absorbance was read at 570 nm using an ELISA microplate reader (Molecular Devices, Sunnyvale, USA).

Flow cytometry analysis SK-28 cells were seeded in 6-well plates and cultured with 0.1% DMSO or various concentrations of PLAB (0.1, 1.0, and 10.0 μmol/L) for 24 h. Following incubation, the cells were collected and fixed with 70% ethanol. These samples were treated with RNase A (10 μg/mL), stained with PI (50 μg/mL) and analyzed with the FACS Calibur flow cytometer (Becton Dickinson, Heidelberg, Germany). All results were obtained from three independent experiments.

Western blot analysis Cytosolic extracts were prepared by suspending 1.0×10^7 cells in 1 mL of ice-cold lysis buffer [50 mmol/L Tris–HCl, pH 7.4, 0.25 mol/L NaCl, 0.1% Nonidet P-40, 5 mmol/L EDTA, 50 mmol/L NaF, 1×cocktail of protease inhibitors (Sigma), 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mg/L aprotinin] and incubated on ice for 30 min. After centrifugation at 8000×*g* for 20 min at 4 °C, the protein in the supernatant was quantified using a protein assay kit from Bio-Rad Laboratories; samples (60 μg) were electrophoresed on 12% SDS–polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were blocked for 90 min at room temperature in PBS plus 0.5% Tween-20 containing 5% fat-free powdered milk, then incubated for 2 h at room temperature with rabbit polyclonal antibodies against human Chk2, Cdc2, Cdc2-P, Cdc25C-P, p21, β-actin, or mouse monoclonal anti-human p53 and p53-p antibodies at concentrations suggested by the manufacturers. After being washed, the membranes were incubated for 90 min at 25 °C with the appropriate horseradish peroxidase-labeled secondary antibody, and bound antibody was visualized and quantified by chemiluminescence detection. β-actin was used as the internal control. The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of β-actin. The density of the band was expressed relative to the density in untreated cells (control, taken as 100%).

ATM kinase assay ATM kinase assays were performed as described previously[14]. Briefly, extracts from 1.0×10^7 cells were prepared by the addition of 500 mL of lysis buffer for 10 min, followed by centrifugation for 15 min at 1500×*g* at 4 °C. The protein content of the supernatant was

estimated by the Bio-Rad method. Samples (100 mg) were incubated for 3 h at 4 °C with 2 mg of monoclonal anti-ATM in a volume of 200 mL; then, 200 mL of protein A-Sepharose was added and the sample was mixed for 2 h at 4 °C on a rotator. The ATM kinase assay was performed for 30 min at 30 °C on the protein A-Sepharose-bound immune complexes resuspended in 500 mL of 10 mmol/L HEPES, pH 7.5, 50 mmol/L β-glycerophosphate, 50 mmol/L NaCl, 10 mmol/L $MgCl_2$, 10 mmol/L $MnCl_2$, 1 mmol/L dithiothreitol, 5 mmol/L ATP, 1 μg of PHAS-1, and 10 mCi $[\gamma\text{-}^{32}P]$ATP. The products were analyzed by SDS–PAGE, followed by autoradiography.

Statistical analysis The results were expressed as the mean±SD and statistically compared with the control group or compared between different drugs using one-way ANOVA and multiple comparison of SAS Base 6.12 software (SAS Inc, USA). Statistically significant differences were reported when $P<0.05$.

Results

Antiproliferative effect of PLAB on A372, SK-28 and 624 mel cells To study the effect of PLAB on human melanoma cells, A372, SK-28 and 624 mel cells were treated with various concentrations of PLAB for 48 h and cell viability was determined by MTT reduction methods. The results indicated that PLAB possessed potent anticancer activity; the concentrations (μmol/L) of 50% cytotoxicity (IC_{50}) against the various human melanoma cell lines were SK-28 (1.12)<A372 (1.93)<624 mel (2.86). The SK-28 cells were chosen for further study and the results demonstrated that treatment with PLAB (0.01–10 μmol/L) resulted in a dose- and time-dependent inhibition of SK-28 cell proliferation. This effect was more pronounced at 24, 48, and 72 h post-treatment compared with that pre-treatment (Figure 2). The inhibition of cell growth might be a result of the induction of cell cycle arrest. Therefore, the mechanism of PLAB-mediated cell cycle perturbation was further evaluated.

PLAB induces cell cycle arrest at the G_2 phase in human melanoma cells Treatment of SK-28 cells with PLAB resulted in a dose- and time-dependent inhibition of cell growth compared with untreated controls at all observed time points. This growth inhibition may involve an arrest of cells at specific checkpoints in the cell cycle. After treatment with PLAB (0.1, 1.0, and 10 μmol/L) for 24 h, an appreciable arrest of cells in G_2/M was observed, accompanied by decreased cell numbers in the G_0/G_1 and S phases (Figure 3).

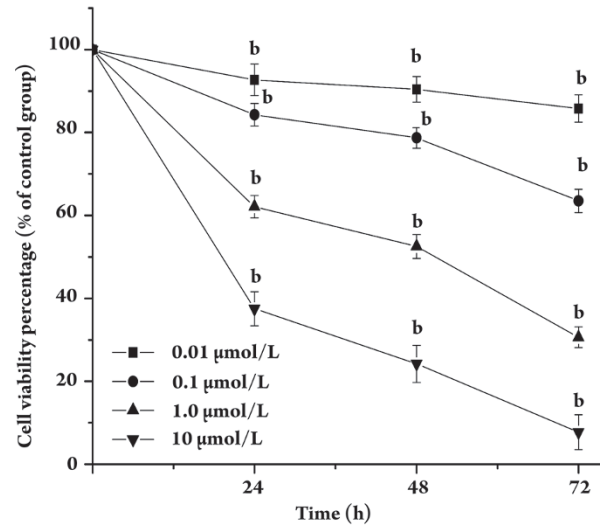**PLAB modulates the expression of G_2/M cell cycle**

Figure 2. Effect of PLAB on the viability of SK-28 cells. The cells were treated with 0.1% DMSO as a control or various concentrations of PLAB for 24, 48, and 72 h, and cell viability was determined by MTT assay. The data represent the mean±SD of three experiments and each conducted in triplicate. $^bP<0.05$ compared with control group.
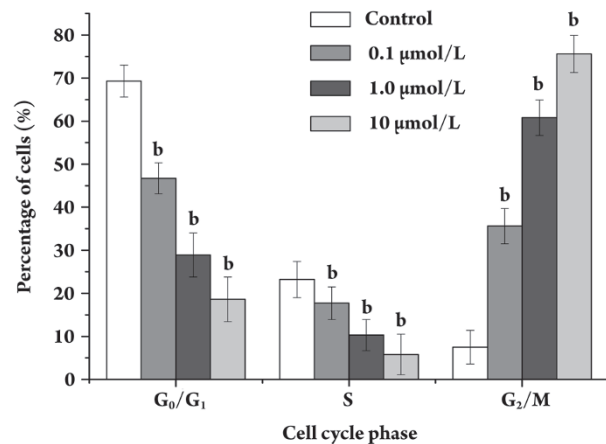

Figure 3. PLAB arrests SK-28 cells at G_2/M phase. The cells (1.5×10^4) were treated with 0.1% DMSO as a control or various concentrations of PLAB (0.1, 1.0, and 10 μmol/L) for 24 h, and then the cell cycle distribution was determined by flow cytometry. The data represent the mean±SD of three experiments and each conducted in triplicate. $^bP<0.05$ compared with control group.

regulatory proteins Cdc2, Cyclin B1, Cdc25C and Wee1 Having established that PLAB induced cell cycle arrest, we attempted to characterize the mechanism at the molecular level. To this end, we investigated the effects of PLAB on expression of key regulators of the G_2 to M transition, Cdc2 (Cdk1, p34) and CyclinB1, in SK-28 cells using Western blot analysis. Treatment with PLAB for 4 h had no effect on Cdc2

or Cyclin B1 protein levels. At 24 h after treatment, PLAB (1.0 and 10 μmol/L) inhibited Cdc2 expression in a dose-dependent manner (Figure 4A). Interestingly, PLAB slightly upregulated Cyclin B1 protein levels (Figure 4B), which is consistent with Cyclin B1 expression in PLAB-treated human breast cancer MCF-7 cells[8]. Because cell cycle entry into the mitotic phase is initiated by dephosphorylation of the inhibitory residues Thr14 and Tyr15 on Cdc2, we examined the Cdc2 phosphorylation status by Western blotting. We found that Thr14 and Tyr15 phosphorylation increased in a dose-dependent manner after 4 h of PLAB treatment (Figure 4C), but at 24 h Cdc2 phosphorylation decreased
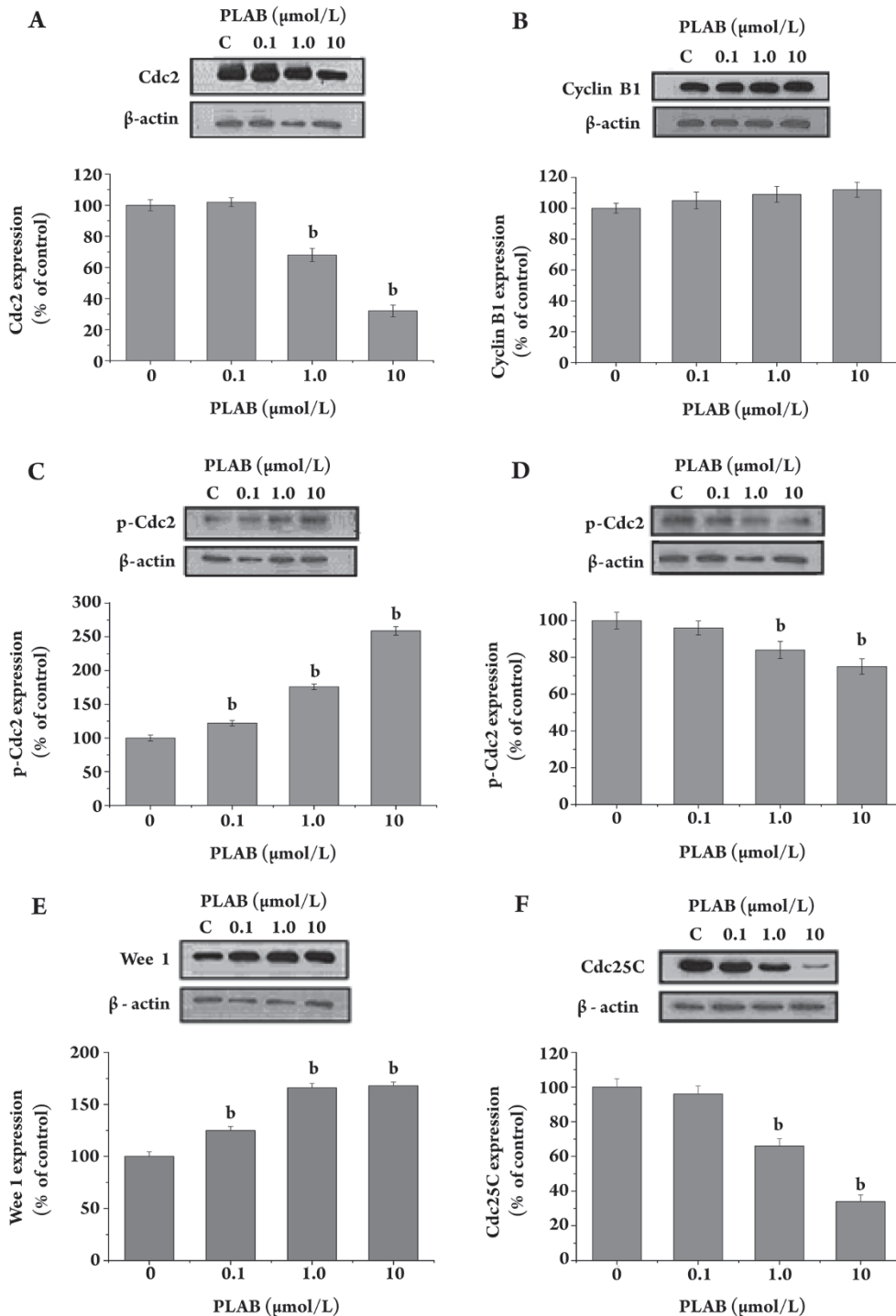

Figure 4. PLAB induces Cdc2, Cyclin B1, p-Cdc2, Wee 1, Cdc25C protein expression in SK-28 cells. The cells (1.0× 10^6) were treated with 0.1% DMSO as a control or various concentrations of PLAB (0.1, 1.0, and 10 μmol/L) for 24 h (A, B, D) or 4 h (C, E, F), then the protein levels of Cdc2, Cyclin B1, p-Cdc2, Wee 1, Cdc25C were evaluated by Western blotting. The data represent the mean± SD of three experiments. [b]$P<0.05$ compared with control group. C, control.

(Figure 4D). The reduced expression of Cdc2 at 24 h and the increased phosphorylation of Cdc2 at 4 h suggest that Cdc2 is a mediator of the G_2/M arrest induced by PLAB.

The Thr14 and Tyr15 residues in the ATP-binding domain of Cdc2 are phosphorylated by Wee1[15, 16] and dephosphorylated by the dual specificity phosphatase Cdc25C[17]. Western blotting showed that Wee1 protein levels were increased in a dose-dependent manner after 4 h of PLAB (1.0 μmol/L) treatment of SK-28 cells (Figure 4E). In contrast, 24 h after exposure to PLAB Wee1 expression was unchanged, coincident with the downregulation in Cdc2 phosphorylation. Because Wee1 expression was enhanced by PLAB at 4 h, we investigated the effect of PLAB on Cdc25C levels and phosphorylation by Western blotting. As shown in Figure 4F, PLAB reduced Cdc25C expression in a dose-dependent manner after exposure of SK-28 cells to PLAB (0.1, 1.0, and 10 μmol/L) for 4 h, whereas only 10 μmol/L of PLAB resulted in increased Cdc25C phosphorylation on Ser216. It is likely that Cdc25C inactivation was due not only to increased phosphorylation but also to decreased nuclear export of active Cdc25C. These data suggest that both increased Wee1 protein expression and decreased Cdc25C levels contribute to the increased Cdc2 phosphorylation seen following PLAB treatment.

PLAB increases p53 expression and the effect is inhib- **ited by caffeine, an ATM kinase inhibitor** The rapid phosphorylation of Cdc2 following treatment with PLAB, which is a typical response of cells to DNA damaging agents[18], led us to speculate that PLAB might cause DNA damage. The tumor suppressor protein, p53, plays a critical role in regulating cell cycle progression after DNA damage[19]. Because SK-28 cells express wild-type p53, we examined possible changes in protein expression induced by PLAB treatment. Indeed, the level of p53 expression increased after a 24-h incubation with PLAB in a dose-dependent manner (0.1, 1.0, and 10 μmol/L, Figure 5A). Phosphorylation of p53 at residue Ser15 plays a role in stabilizing p53 and enhancing its trans-activation capacity[19]. Accordingly, treatment with PLAB for 4 h resulted in a concentration-dependent elevation in the phosphorylation of p53 at Ser15, whereas no phosphorylation was detected in control cells (Figure 5B).

Phosphorylation of p53 at Ser15 is usually catalyzed by the protein kinases ataxia telangiectasia mutated (ATM) and ataxia telangiectasia-Rad3-related (ATR)[20, 21]. Caffeine has been demonstrated to inhibit the activity of these kinases[22]. Thus, in order to gain preliminary data concerning the possible connection between the observed PLAB-induced p53 phosphorylation and activation of the kinases ATM/ATR in SK-28 cells, we treated the cells with caffeine (0, 3, and 5 mmol/L) prior to PLAB treatment. Caffeine-treated cells
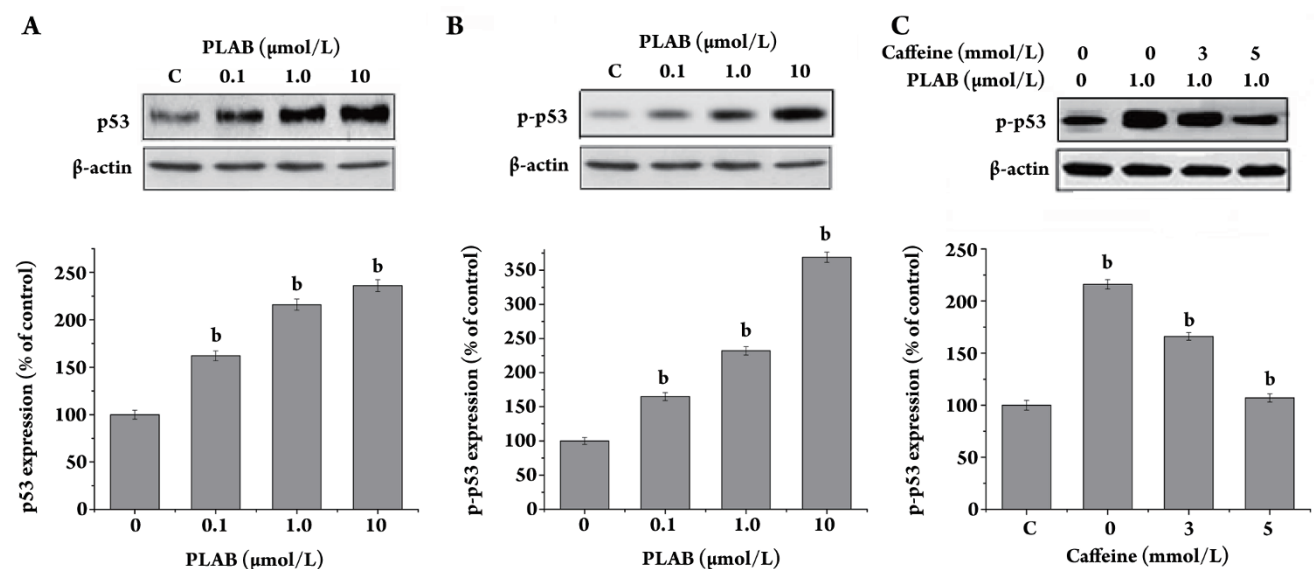

Figure 5. PLAB increases the protein expression of p53 and p-p53 in SK-28 cells and the effect on p-p53 protein is blocked by caffeine. The cells (1.0×10^6) were treated with 0.1% DMSO as a control or various concentrations of PLAB (0.1, 1.0, and 10 μmol/L) (A and B) or with PLAB (1.0 μmol/L) and 0, 3, or 5 mmol/L caffeine (C), then p53 and p-p53 protein levels were evaluated by Western blotting. The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of β-actin, then the density of the band was expressed as the relative density compared to that in untreated cells (control), taken as 100%. The data represent the mean±SD of three experiments. $^b P<0.05$ compared with control group. C, control.

exhibited a significant decrease in Ser15 phosphorylation (Figure 5C), suggesting that ATM/ATR may be involved in activation of p53 by PLAB.

PLAB increases p21$^{waf1/cip1}$ expression One of the target genes of p53 is p21$^{waf1/cip1}$, a cyclin-dependent kinase inhibitor (CDKI) that inhibits the activity of Cdc2-Cyclin B1 complexes[23]. In order to verify the transactivation activity of p53 following treatment with PLAB and to determine whether p21$^{waf1/cip1}$ was involved in the PLAB-induced reduction in Cdc2 activity, p21$^{waf1/cip1}$ protein expression was analyzed. Western blots of extracts of cells treated for 24 h with PLAB (0.1, 1.0, and 10 μmol/L) showed a marked increase in p21$^{waf1/cip1}$ protein levels, which was negatively correlated with Cdc2 activity (Figure 6). These results suggest that PLAB induces p53-dependent p21$^{waf1/cip1}$ expression, and induction of p21$^{waf1/cip1}$ expression might account for a large part of the reduction in Cdc2 activity, leading to the observed early accumulation of cells in G$_2$/M phase.
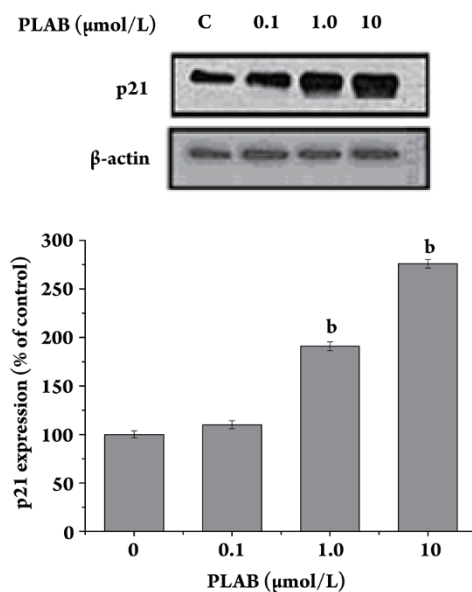

Figure 6. PLAB increases p21$^{waf1/cip1}$ protein expression. The cells (1.0×10^6) were treated with 0.1% DMSO as a control or various concentrations of PLAB (0.1, 1.0, and 10 μmol/L) for 24 h, and then p21$^{waf1/cip1}$ protein levels were evaluated by Western blotting. The data represent the mean±SD of three experiments. ^{b}P<0.05 compared with control group. C, control.

PLAB increases Chk2 expression and the effect is inhibited by caffeine, an ATM kinase inhibitor Checkpoint kinases can phosphorylate Cdc25C on Ser216 by an ATM-dependent pathway[24, 25]. To determine whether the inactivation of Cdc25C is due to ATM-dependent Chk2 acti-

vation, we examined the effect of PLAB on the expression of Chk2, the major target of ATM kinase[14], and the effect of caffeine, an ATM kinase inhibitor[22, 26]. When SK-28 cells were exposed to PLAB (0.1–10 μmol/L) for 4 h, Chk2 levels showed a dose-dependent increase (Figure 7A), which was blocked by pre-treatment of cells with caffeine (0, 3, and 5 mmol/L, Figure 7B). These results suggest that PLAB induced ATM-mediated Chk2 expression in SK-28 cells.

PLAB enhances ATM kinase activity SK-28 cells were treated with PLAB (0.1, 1.0, and 10 μmol/L) for 4 h and ATM kinase activity was assayed using the specific substrate, PHAS-1. ATM kinase activity was significantly increased after 4 h treatment with PLAB (10 μmol/L, Figure 8).

Discussion

In this study, we demonstrated that PLAB had a broad spectrum of activity against human melanoma cells, and we further investigated PLAB's mechanism of action using the human SK-28 cell line as a representative model. We found that PLAB caused a dramatic accumulation of cells in G$_2$ phase of the cell cycle in SK-28 cells, accompanied by up-regulation of Cdc2 phosphorylation and subsequent down-regulation of Cdc2 expression. Furthermore, PLAB decreased the expression of Cdc25C phosphatase and increased the expression of Wee1 kinase. Meanwhile, reduction in Cdc2 expression was partly due to induction of the expression of p21$^{waf1/cip1}$. In addition, PLAB activated the checkpoint kinase Chk2 and increased the expression of p53, two major targets of ATM kinase. These effects were inhibited by caffeine, an ATM kinase inhibitor. We also found that PLAB significantly enhanced ATM kinase activity. Although activation of Cdc2 by PLAB has been shown in AGS human gastric cancer cells[13], ours is the first study to demonstrate that PLAB induced G$_2$/M arrest in human melanoma cells via a mechanism involving the activation of ATM. We further showed that the effect of PLAB on Cdc2 activity is mediated by interactions with the Chk2-Cdc25C and p53 signaling pathways, two distinct downstream pathways of ATM.

The role of the G$_2$/M checkpoint is to allow cells to repair DNA damage before entering mitosis so the number of DNA lesions passed on to the daughter cells is minimized. Therefore, the G$_2$/M checkpoint plays a key role in the maintenance of chromosomal integrity. A key regulator of the cell cycle at this checkpoint is Cdc2 kinase[27]. Cdc25C phosphatase and Wee1 kinase are responsible for the dephosphorylation and phosphorylation of Cdc2, respectively; without functional Cdc25C, Cdc2 remains phosphorylated and unable to form an active complex with Cyclin B1, resulting
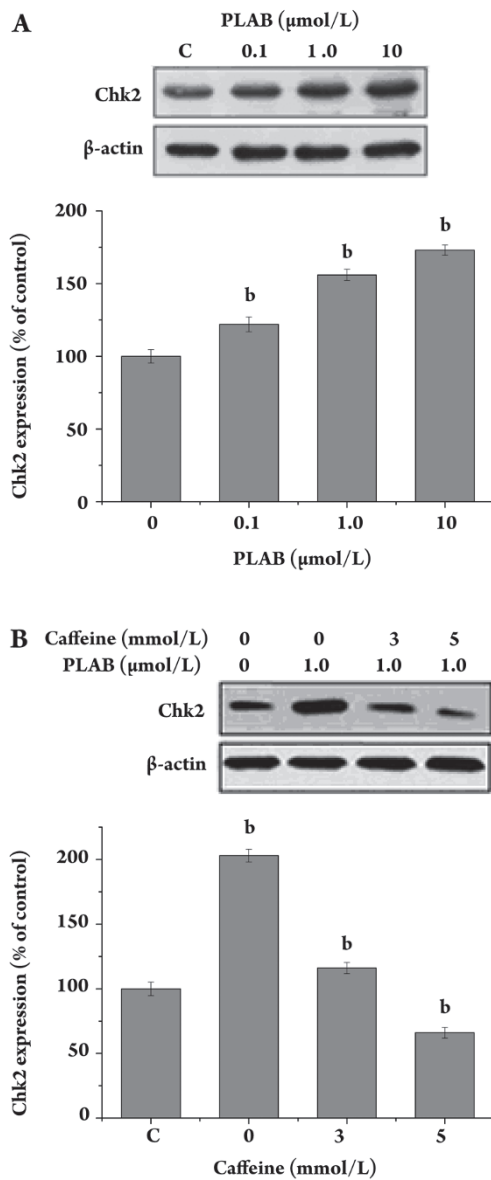
A

B

Figure 7. PLAB increases Chk2 protein expression in SK-28 cells and the expression is blocked by caffeine. The cells (1.0×10^6) were treated with 0.1% DMSO as a control or various concentrations of PLAB (0.1, 1.0, and 10 μmol/L) (A) or with PLAB (1.0 μmol/L) and 0, 3, or 5 mmol/L caffeine (B) for 4 h, then Chk2 protein levels were evaluated by Western blotting. The amount of the protein of interest, expressed as arbitrary densitometric units, was normalized to the densitometric units of β-actin, then the density of the band was expressed as the relative density compared to that in untreated cells (control), taken as 100%. The data represent the mean±SD of three experiments, $^{b}P<0.05$ compared with control group. C, control.

Figure 8. PLAB increases ATM kinase activity in SK-28 cells. The cells (1.0×10^6) were treated with 0.1% DMSO as a control or various concentrations of PLAB (0.1, 1.0, and 10 μmol/L) for 4 h, then ATM was immunoprecipitated and kinase activity assayed using PHAS-1 as substrate, followed by SDS–PAGE and autoradiography. The data represent the mean±SD of three experiments and each conducted in triplicate. $^{b}P<0.05$ compared with control group. C, control.

24 h. We also examined Cdc2 phosphorylation status and found an elevation in the phosphorylation of Cdc2 (p-Cdc2) after 4 h of PLAB treatment. Interestingly, at 24 h a decrease in Cdc2 phosphorylation was observed, probably due to decreased expression or increased degradation of Cdc2 itself. Moreover, we found that Cdc2 phosphorylation was mediated by PLAB-mediated decreased Cdc25C levels and increased Wee1 levels. Additionally, Cdc25C inactivation was due not only to increased phosphorylation but also to decreased nuclear export of active Cdc25C. In summary, our observations of increased Cdc2 phosphorylation followed by reduction in Cdc2 expression suggest that PLAB-induced G_2/M phase arrest in SK-28 melanoma cells is mediated by inhibition of Cdc2 activity.

The tumor suppressor proteins Chk1 and Chk2 play important roles in regulating the G_2/M checkpoint and are closely related to serine/threonine protein kinases that are capable of phosphorylating a number of proteins in response to DNA damage. At 24 h after PLAB treatment, a dose-dependent increase of Chk2 expression was observed. Because Chk2 has been linked to G_2 cell cycle arrest through its ability to inhibit Cdc25C[28], it is likely that the phosphorylation of Cdc25C phosphatase occurs as a result of upstream activation of Chk2 by PLAB. Indeed, we observed that 10 μmol/L PLAB resulted in increased Cdc25C phosphorylation,

in cell cycle arrest in the G_2 phase. In this study, we monitored Cdc2 status 4 h and 24 h after treatment with PLAB. No change was found in Cdc2 levels at 4 h, but a decrease in Cdc2 levels in a PLAB dose-dependent manner was seen at
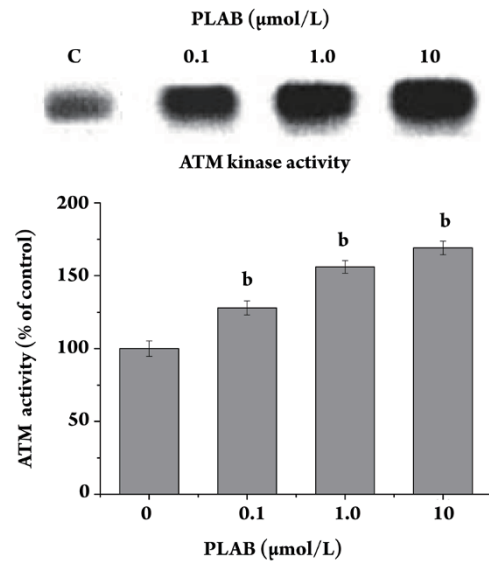
suggesting that exposure of SK-28 human melanoma cells to PLAB may cause activation of Chk2 and subsequent phosphorylation of Cdc25C phosphatase. Cdc25C phosphorylation, in turn, results in the phosphorylation/inhibition of Cdc2, a critical event for the correct functioning of the Cdc2-cyclin B1 complex. Because ATM phosphorylation of N-terminal sites in Chk2[29] is important for signaling DNA damage to cell cycle checkpoints, we examined the effect of caffeine, an ATM kinase inhibitor, on the above effect. Our finding is that the above effect was blocked by caffeine. The effect of caffeine is due to inhibition of the protein kinase activities of ATM and the ATM- and Rad3-related kinase (ART)[30]. In addition, we showed that PLAB increased ATM activity, which was also abrogated by caffeine. Therefore, we propose that the PLAB-induced cell cycle arrest at G_2/M phase in SK-28 cells may require activation of Chk2 in an ATM-dependent manner.

ATM, the gene mutated in ataxia-telangiectasia, encodes a 370-kDa protein that is a member of a family of proteins related to PI3-K and is known to phosphorylate p53 at Ser15 in response to DNA damage[31]. The tumor suppressor protein p53 negatively regulates Cdc2 activity and inhibits the transcription of Cyclin B1/Cdc2[32]. We found that p53 expression increased after a 24 h incubation with PLAB in a dose-dependent manner. Furthermore, phosphorylation of p53 showed a clear concentration-dependent elevation. The increased phosphorylation of p53 at Ser15 following treatment with PLAB became almost undetectable in cells pretreated with caffeine. The findings presented here suggest that ATM and/or ATR might be involved in the signaling pathway initiated by PLAB in melanoma cells. One of the transcription targets of p53 is the tumor suppressor protein p21[waf1/cip1], which acts as an inhibitor of cell cycle progression via its ability to inhibit Cdc2[33]. Therefore, in addition to higher levels of p21[waf1/cip1] in PLAB-treated SK-28 cells compared with untreated cells, PLAB may also inhibit Cdc2 due to its action on p53 and downstream p21[waf1/cip1]. Moreover, the PLAB-induced p53 accumulation and increased p21[waf1/cip1] expression were attenuated by caffeine, suggesting that p53-dependent p21[waf1/cip1] expression might occur in PLAB-treated SK-28 cells.

As discussed above, we suggest that PLAB-induced arrest in the G_2 phase of the cell cycle was due to activation of Chk2 and p53 in an ATM-dependent manner. Moreover, we showed that PLAB increased ATM activity, but ART activity was not examined, so it is possible that protein kinases other than Chk2 are also activated by PLAB treatment.

Based on the outcome of this study and the available literature, we suggest multiple pathways by which PLAB results

in cell cycle arrest. This may involve (1) inhibition of Cdc2 activation/dephosphorylation by PLAB-induced decreases in Cdc25C levels and increases in Wee1 levels, (2) down-regulation of Cdc2 activity by PLAB-mediated induction of p21[waf1/cip1] expression in a p53-dependent manner, (3) reduction of Cdc25 expression by ATM-dependent Chk2 activation, and/or (4) PLAB-induced up-regulation of p53 in an ATM-dependent manner (Figure 9). We demonstrated that PLAB may be a promising chemopreventive agent for treating human melanoma.
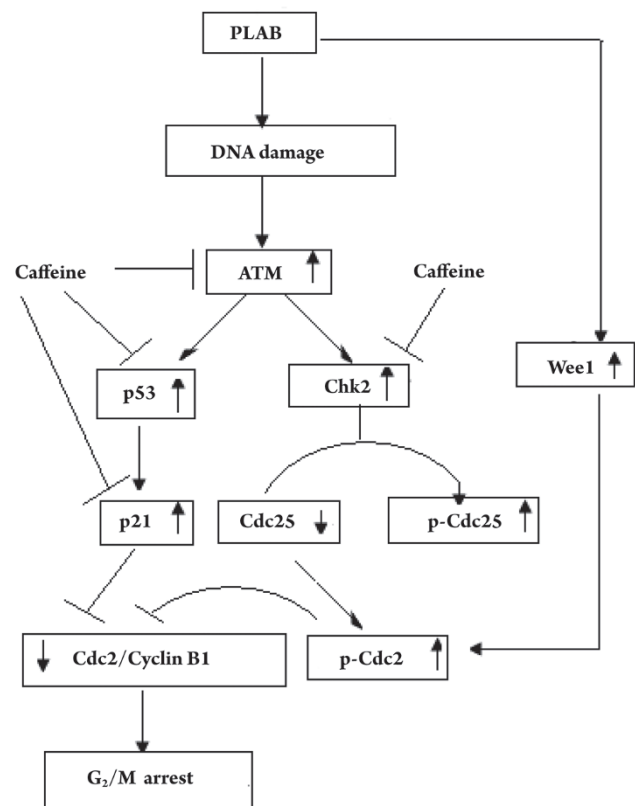

Figure 9. Putative mechanism of PLAB-induced cell cycle G_2/M arrest in human melanoma cells. PLAB might cause DNA damage which activates ATM kinase. This activation possibly increases the expression of p53 and Chk2. An increment of p53 in cells causes transactivate p21[waf1] and an increment of Chk2 phosphorylates/inactivates Cdc25C. Caffeine, an ATM kinase inhibitor, blocks the effects of PLAB on Chk2 and p53. PLAB might result in up-regulation of Wee1. An increment of Wee1, a decrement of Cdc25C, and an elevation of p21[waf1] might cause inhibition of Cdc2, which contributes to G_2/M arrest. (↑) increased; (↓) decreased; (⊥) abolished.

Acknowledgements

References

1 Li EI, Clark A, Hufford C. Antifungal Evaluation of Pseudolaric Acid B, a Major Constituent of Pseudolarix kaempferi. J Nat Prod 1995; 58: 57–67.

2 Yang SP, Dong L, Wang Y, Wu Y, Yue JM. Antifunal diterpenoids of Pseudolarix kaempferi, and their structure activity relationship study. Bioorg Med Chem 2003; 11: 4577–84.

3 Wang WC, Lu RF, Zhao SX, Zhu YZ. Antifertility effect of pseudolaric acid B. Acta Pharmacol Sin 1982; 3: 188–92. Chinese.

4 Zhang YL, Lu RZ, Yan AL. Inhibition of ova fertilizability by pseudolaric acid B in hamster. Acta Pharmacol Sin 1990; 11: 60–2. Chinese.

5 Wang WC, Gu ZP, Koo A. Chen WS. Effects of pseudolaric acid B on blood flows of endometrium and myometrium in pregnant rats. Acta Pharmacol Sin 1991; 12: 423–5. Chinese.

6 Tan WF, Zhang XW, Li MH, Yue JM, Chen Y, Lin LP, *et al*. Pseudolaric acid B inhibits angiogenesis by antagonizing the vascular endothelial growth factor-mediated anti-apoptotic effect. Eur J Pharmacol 2004, 499: 219–28.

7 Li MH, Miao ZH, Tan WF, Yue JM, Zhang C, Lin LP, *et al*. Pseudolaric acid B inhibits angiogenesis and reduces hypoxia-inducible factor lalpha by promoting proteasome-mediated degradation. Clin Cancer Res 2004, 10: 8266–74.

8 Yu JH, Cui Q, Jiang YY, Yang W, Tashiros, Onodera S, *et al*. Pseudolaric acid B induces apoptosis, senescence, and mitotic arrest in human breast cancer MCF-7. Acta Pharmacol Sin 2007; 28: 1975–83.

9 Ko JK, Leunq WC, Ho WK, Chiu P. Herbal diterpenoids induce growth arrest and apoptosis incolon cancer cells with increased expression of the nonsteroidal anti-inflammatory drug-activated gene. Eur J Pharmacol 2007; 559: 1–13.

10 Gong X, Wang M, Wu Z, Tashiro S, Onodera S, Ikejima T. Pseudolaric acid B inducesapoptosis via activation of c-Jun N-terminal kinase and caspase-3 in HeLa cells. Exp Mol Med 2004; 36: 551–6.

11 Gong XF, Wang MW, Tashiros, Onodera S, Ikejima T. Pseudolaric acid B induces apoptosis through p53 and Bax/Bcl-2 pathways in human melanoma A375-S2 cells. Arch Pharm Res 2005; 28: 68–72.

12 Yu JH, Wang HJ, Li XR, Tashiro SI, Onodera S, Ikekima T. Protein tyrosine kinase, JNK, and ERK involvement in pseudolaric acid B-induced apoptosis of human breast cancer MCF-7 cells. Acta Pharm Sin 2008; 29: 1069–76.

13 Li KS, Gu XF, Li P, Zhang Y, Zhao YS, Yao ZJ, *et al*. Effect of pseudolaric acid B on gastric cancer cells: inhibition of proliferation and induction of apoptosis. World J Gastroenterol 2005; 11: 7555–9.

14 Ye R, Bodero A, Zhou BB, Khanna KK, Lavin MF, Lees-Miller SP. The plant isoflavonoid genistein activates p53 and Chk2 in an ATM-dependent manner. J Biol Chem 2001; 276: 4828–33.

15 McGowan CH, Russell P. Human Wee1 kinase inhibits cell division by phosphorylating p34cdc2 exclusively on Tyr15. EMBO J 1993, 12: 75–85.

16 Booher RN, Holman PS, Fattaey A. Human Myt1 is a cell cycle regulated kinase that inhibits Cdc2 but not Cdk2 activity. J Biol Chem 1997; 272: 22300–6.

17 Jin P, Gu Y, Morgan DO. Role of inhibitory CDC2 phosphorylation in radiation-induced G2 arrest in human cells. J Cell Biol 1996; 134: 963–70.

18 Nurse P. Checkpoint pathways come of age. Cell 1997; 91: 865–7.

19 Vousden KH, Lu X. Live or let die: the cell's response to p53. Nat Res Cancer 2002; 2: 594–604.

20 Banin S, Moyal L, Shieh S, Taya Y, Anderson CW, Chessa L, *et al*. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science 1998; 281: 1674–7.

21 Canman CE, Lim DS. The role of ATM in DNA damage responses and cancer. Oncogene 1998; 17: 3301–8.

22 Sarkaria JN, Busby EC, Tibbetts RS, Roos P, Taya Y, Karnitz LM, *et al*. Inhibition of ATM and ATR kinase activities by the radiosensitizing agent, caffeine. Cancer Res 1999; 59: 4375–82.

23 Deveraux QL, Reed JC. IAP family proteins — suppressors of apoptosis. Genes Dev 1999; 13: 239–52.

24 Peng CY, Graves PR, Thoma RS, Wu Z, Shaw AS, Piwnica-Worms H. Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science 1997; 277: 1501–5.

25 Sanchez Y, Wong C, Thoma RS, Richman R, Wu Z, Piwnica-Worms H, *et al*. Conservation of the Chk1 checkpoint pathway in mammals: linkage of DNA damage to Cdk regulation through Cdc25. Science 1997; 277:1497–501.

26 Blasina A, Price BD, Turenne GA, McGowan CH. Caffeine inhibits the checkpoint kinase ATM. Curr Biol 1999; 9: 1135–8.

27 Li L, Zou L. Sensing, signaling, and responding to DNA damage: organization of the checkpoint pathways in mammalian cells. J Cell Biochem 2005; 94: 298–306.

28 O'Connor PM, Ferris DK, Hoffmann I, Jackman J, Draetta G, Kohn KW. Role of the cdc25C phosphatase in G2 arrest induced by nitrogen mustard. Proc Natl Acad Sci USA 1994; 91: 9480–4.

29 Chan DW, Son SC, BlockW, Ye R, Douglas P, Goodarzi AA, *et al*. Purification and characterization of ATM from human placenta. a manganese-dependent, wortmannin-sensitive serine/threonine protein kinase. J Biol Chem 2000; 275: 7803–10.

30 Darbon JM, Penary M, Escalas N, Casagrande F, Goubin-Gramatica F, Baudouin C, *et al*. Distinct Chk2 activation pathways are triggered by genistein and DNA-damaging agents in human melanoma cells. J Biol Chem 2000; 275: 15363–9.

31 Canman CE, Lim DS. The role of ATM in DNA damage responses and cancer. Oncogene 1998; 17: 3301–8.

32 Yun J, Chae HD, Choy HE, Chung J, Yoo HS, Han MH, *et al*. p53 negatively regulates cdc2 transcription via the CCAAT-binding NF-Y transcription factor. J Biol Chem 1999; 274: 29677–82.

33 Dash BC, EI-Deiry WS. Phosphorylation of p21 in G_2/M promotes cyclin B-Cdc2 kinase activity. Mol Cell Biol 2005; 25: 3364–87.