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Paclitaxel increases p21 synthesis and accumulation of its AKT-phosphorylated form in the cytoplasm of cancer cells

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CKI p21 is a regulator of cellular responses to microtubule damage induced by drugs such as paclitaxel (PTX). It mediates the G1 4N arrest postactivation of the spindle assembly checkpoint and protects cancer cells against PTX-induced cytotoxicity. We demonstrated here that low doses of PTX that are unable to activate the spindle assembly checkpoint, upregulate p21 by a p53-dependent pathway and induce its translocation to the cytoplasm. This cytoplasmic accumulation of p21 resulted from an AKT-dependent p21 phosphorylation leading to an association of p21 with 14-3-3. Furthermore, the cytoplasmic p21 accumulation observed in PTX-treated cells was inhibited by LY 294002, a specific PI-3 kinase inhibitor or by the expression of a dominant-negative AKT mutant. However, the kinase activity of AKT was unchanged in PTX-treated cells, suggesting that low doses of PTX could regulate p21 phosphorylation via inhibition of its dephosphorylation. As a functional consequence, we found that cytoplasmic accumulation of the phosphorylated form of p21 prevents the inhibitory effect of p21, enabling these cells to escape to the p53-dependent G1/S and G2/M checkpoints.
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Introduction

The cyclin-dependent kinase (CDK) inhibitor p21 is a universal inhibitor of cell cycle progression (Sherr and Roberts, 1995). Several recent studies have shown that p21 is also a positive modulator of cell survival (Gorospe *et al*., 1999). Expression of p21 is induced in a p53-dependent manner in response to DNA-damaging drugs resulting in a G1/S cycle arrest (Harper *et al*., 1993). Cells deficient in p21 display a greater sensitivity to DNA-damaging agents (Waldman *et al*., 1996, 1997). Microtubule damage activates the mitotic spindle checkpoint, which results in a transient arrest of the cell cycle in mitosis. Following mitotic arrest, cells can either enter apoptosis or exit mitosis without dividing and enter a tetraploid, multinucleated G1 state. A p53-dependent induction of p21 is required for cell cycle arrest in G1 4N after activation of the spindle assembly, preventing cells from endoreplication (Khan and Wahl, 1998; Lanni and Jacks, 1998; Stewart *et al*., 1999a). However, it has been recently shown that p21 can be induced by low concentrations of paclitaxel (PTX), which are insufficient to activate the spindle assembly checkpoint (Torres and Horwitz, 1998; Giannakakou *et al*., 2001). Although the p53 signaling pathway induced by DNA damage has been identified, the pathway elicited by microtubule damage and leading to increase in p53 and p21 has yet to be elucidated. A recent study has shown that p53 is phosphorylated after microtubule damage, but the pattern of p53 phosphorylation is distinct from that observed after DNA damage (Stewart *et al*., 2001). After inactivation of p53 by transfection of human papilloma virus E6 gene, PTX was unable to increase levels of p21 protein (Vikhanskaya *et al*., 1998). An involvement of Raf kinase signaling in induction of p21 by microtubule-active drugs has been described (Blagosklonny *et al*., 1995), although doubt has been cast on this result by the observation that p53 and p21 can be increased by doses of PTX which are unable to activate Raf-1 kinase (Torres and Horwitz, 1998). AKT, a known inhibitor of apoptosis, has been recently shown to be involved in p21 regulation. p21 is phosphorylated at Thr145 and Ser146 by AKT (Li *et al*., 2001) and these phosphorylations appear to regulate p21 function (Li *et al*., 2001; Rossig *et al*., 2001; Zhou *et al*., 2001). However, different molecular mechanisms are now thought to be involved in the regulation of p21 function by phosphorylation. First, in HER-2/neu overexpressing cells, phosphorylation of p21 on Thr145 results in its cytoplasmic accumulation (Zhou *et al*., 2001). Second, this AKT-dependent phosphorylation of p21 prevents the formation of a complex between p21 and PCNA, decreases binding of p21 to CDK2 and promotes the assembly of cyclin D1/CDK4 complex (Li *et al*., 2001; Rossig *et al*., 2001). Third, the phosphorylation of p21 on Ser146 by AKT regulates p21 stability (Li *et al*., 2001). Several recent studies argue in favor of a role of the PI-3 kinase/AKT survival pathway in the regulation of p21 induced by PTX. Indeed, induction of p21 confers resistance to PTX-mediated cytotoxicity (Barboule *et al*., 1997; Yu

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et al., 1998; Schmidt *et al*., 2000) and upregulation of p21 plays a role in AKT-dependent survival after PTX treatment (Li *et al*., 2001). Furthermore, inactivation of the PI-3 kinase/AKT pathway either by its specific inhibitor LY 294002 or by expression of a dominant-negative mutant of AKT has been shown to reduce PTX-induced expression of p21 (Mitsuuchi *et al*., 2000; Li *et al*., 2001).

We here report that low doses of PTX induce both an increase of p21 synthesis by a p53-dependent pathway and the cytoplasmic accumulation of p21 via regulation of its AKT-dependent phosphorylated status, resulting in an inhibition of its antiproliferative effect.

Results

Low doses of PTX induce a p53-dependent synthesis of p21, which occurs independently of the spindle assembly checkpoint

It has been shown recently that p21 is induced by PTX at lower doses than those required to block the cell cycle in mitosis (Torres and Horwitz, 1998; Giannakakou *et al*., 2001). Our results indicate that this is also the case in RPMI-2650 cells: p21 protein levels peaked after addition of 10 nM PTX (Figure 1a), whereas a dose of 50 nM PTX was required to detect the presence of the phosphorylated form of histone H1, a marker of mitosis (Figure la) or an accumulation of cells with a G2/M DNA content (Figure 1a). Microtubule staining with anti-alpha tubulin indicated that in control and PTX-treated cells (10 nM), mitotic cells contain bipolar spindles (Figure 1b). DAPI staining indicated that cells treated with 10 nM PTX progress through anaphase (Figure 1b) and telophase (data not shown). In contrast, following treatment with 100 nM PTX, most cells accumulated in an abnormal mitotic phase (DAPI staining) and contained clusters of microtubules (tubulin staining) (Figure 1b).

With low doses of PTX, the induction of p21 was dependent on p53 as was observed when RPMI-2650 cells were transfected by the human papilloma virus E6 gene, which inactivates p53 and inhibits PTX-induced p21 accumulation (Figure 1c). As a positive control, the γ irradiation of E6-transfected RPMI-2650 cells did not increase levels of p21 (Figure 1c).

Taken together, these observations indicate that PTX-induced p21 expression is independent of activation of the spindle assembly checkpoint in this cancer cell line.

Induction of p21 by low doses of PTX is not associated with inhibition of cell proliferation

Treatment of murine and rat fibroblasts with microtubule-active drugs leads to a p53-dependent accumulation of p21 followed by cell cycle arrest at the G1/S phase boundary (Trielli *et al*., 1996; Sablina *et al*., 2001). It has recently been shown that in A549 cells, low doses of PTX, unable to activate the spindle assembly checkpoint, induce p21 and G1 and G2 arrests thereby inhibiting cell proliferation (Giannakakou *et al*., 2001). The increase in p21 in PTX-treated (10 nM) RPMI-2650 cells was not associated with any significant alteration in cell cycle parameters in contrast to those treated with 100 nM (Figure 2a). Furthermore, dual analysis of p21

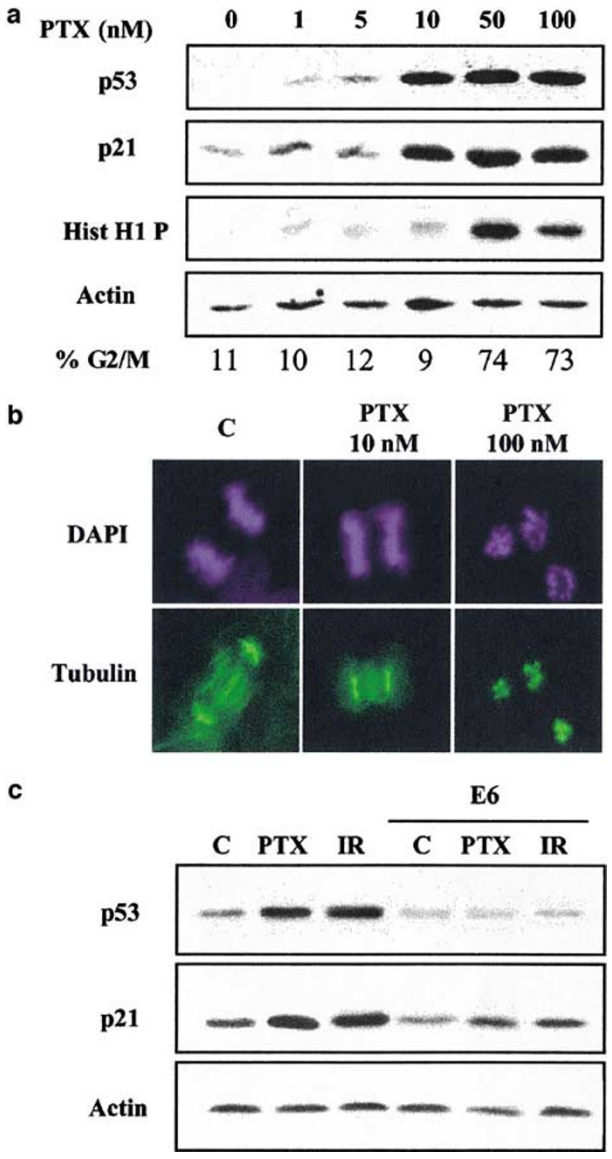


Figure 1 Low doses of PTX induce a p53-dependent upregulation of p21. (**a**) RPMI-2650 cells were treated with various concentrations of PTX for 24 h. Cells were harvested and p53, p21 and the phosphorylated form of histone H1 protein levels were analysed by Western blot. The percentage of cells with a G2/M DNA content was determined by flow cytometry after propidium iodide staining. (**b**) Low doses of PTX are unable to arrest the cell cycle in mitosis. Cells treated or not with PTX (10 and 100 nM) for 24 h were fixed, labeled with tubulin α antibody and DAPI. (**c**) Effect of E6 transfection on p53 and p21 protein levels in PTX-treated cells. RPMI-2650 cells were transiently transfected with a vector allowing the expression of the human papilloma virus E6 protein (transfection efficiency was 50%). At the end of transfection, cells were treated with PTX (10 nM) or γ irradiation (12 grays) and harvested, respectively, 24 and 3 h later, p53 and p21 protein levels were analysed by Western blot on total cell extracts

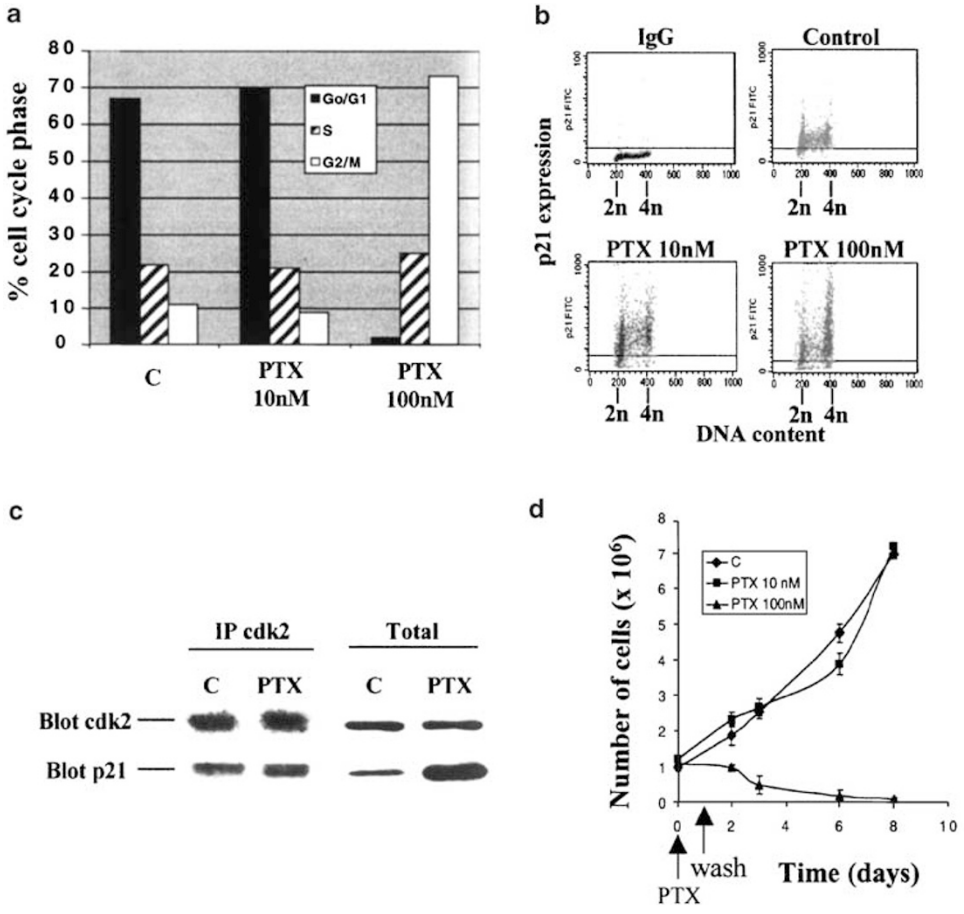


Figure 2 Low doses of PTX do not affect cancer cell proliferation, (**a**) Effect of PTX on RPMI-2650 cell cycle parameters. The percentage of cells in the different phases of the cell cycle were analysed by flow cytometry. (**b**) Dual analysis of p21 expression and DNA content of control and PTX-treated cells. (**c**) Association of p21 with CDK2. Cell extracts were immunoprecipitated with CDK2 antibody. p21 was detected by Western blot analysis of CDK2 precipitates. (**d**) Effect of a 24 h treatment with PTX (10 and 100 nM) on RPMI-2650 cell proliferation. After 24 h, cells were washed and maintained in culture medium for an additional 3 days. Cells were then counted. Results represent the mean ± s.d. of three determinations

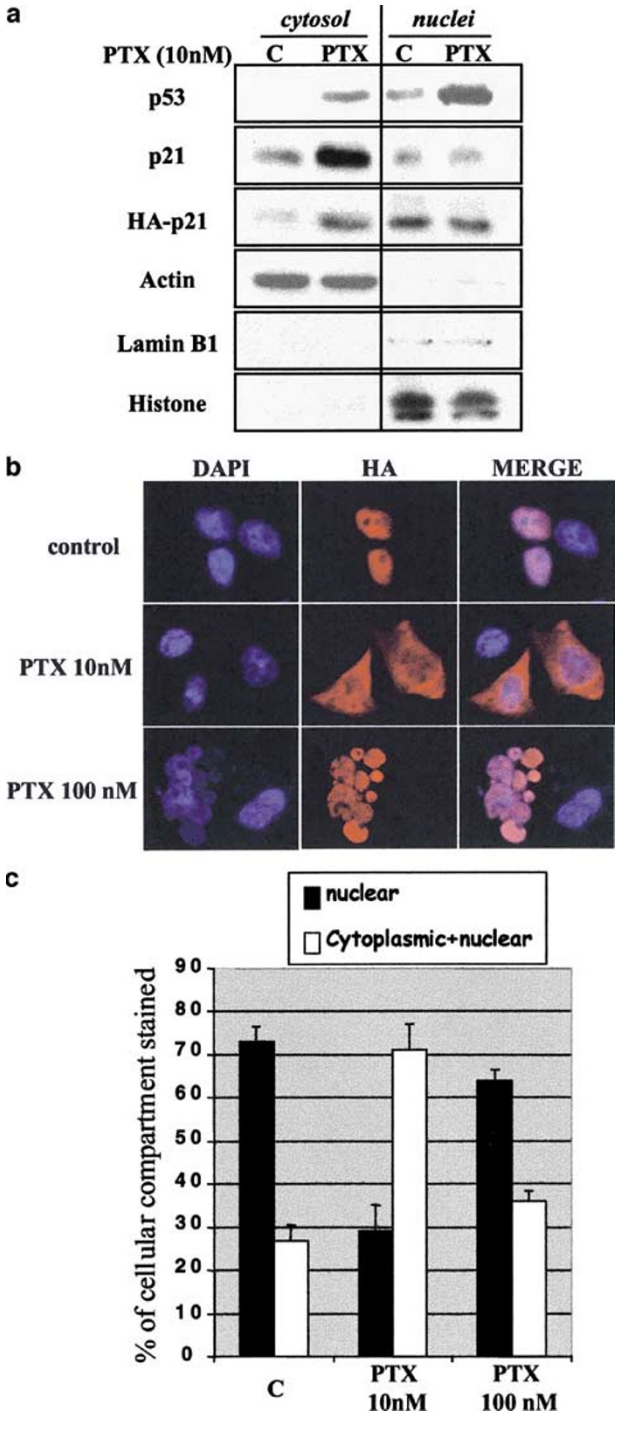
protein and DNA contents of control and PTX-treated cells indicated that p21 was elevated in cells treated with low doses of PTX irrespective of cell cycle phase (Figure 2b), and that the distribution of cells in all phases of the cell cycle was not affected by PTX (Figure 2a, b). The inability of p21 to block the cell cycle of PTX-treated cells was consistent with the observation that the association of p21 to CDK2 was identical to that in control cells (Figure 2c), although levels of p21 were increased. The fact that PTX does not modify cell cycle distribution suggested that either the PTX-treated cells were blocked in all phases of the cell cycle or that p21 was unable to affect cell proliferation. Consequently, we counted the number of RPMI-2650 cells 96 h after addition of a low (10 nM) or a high (100 nM) concentration of PTX. In order to avoid cellular accumulation of PTX, this drug was added during the first 24 h. We first verified that, under these conditions, p21 was maintained at a maximal level between 24 and 96 h (data not shown). As shown in Figure 2d, proliferation of RPMI-2650 cells was inhibited by 100nM PTX, but not affected by 10 nM PTX.

PTX induces p21 accumulation in the cytoplasm

To test whether the loss of p21 function in PTX-treated cells was associated with altered cellular localization, p21 distribution was assessed by subcellular fractionation. As shown in Figure 3a, in control cells, p21 was present in both cytosolic and nuclear fractions, whereas p53 was only detected in nuclei. Incubation of RPMI-2650 cells with a low dose of PTX (10 nM) for 24 h, promoted accumulation of cytosolic p21 with no significant increase in the nuclear fraction. In contrast, we observed that p53 accumulated mainly in the nuclear fraction in PTX-treated cells. We also demonstrated that when p21-tagged with HA epitope (HA-p21) was expressed in RPMI-2650 cells, PTX induces its accumulation in the cytoplasm (Figure 3a).

The p21 distribution was also assessed by immunostaining using an anti-HA antibody. As shown in Figure 3b, c, transfected p21 was present mainly in the nucleus of control RPMI-2650 cells, while it was seen in both cytoplasm and nucleus of PTX-treated cells. After transfection of HA-p21, 75% of control cells presented

nuclear HA staining compared to around 25% of PTX-treated cells (Figure 3c). These results indicate that a low concentration of PTX triggers cytoplasmic accumulation of p21. In contrast, when RPMI-2650 cells were treated with 100 nM PTX, after a transient arrest in mitosis, they entered a tetraploid multinucleated G1 state. In this case, we observed that p21 was localized in the nucleus (Figure 3b).



PTX induces accumulation of the AKT-phosphorylated form of p21

A recent study demonstrated that AKT phosphorylates p21 resulting in its cytoplasmic accumulation (Zhou *et al*., 2001). Furthermore, it has been shown recently that p21 is a mediator of AKT cell survival after treatment with PTX (Li *et al*., 2001). In order to determine if the PI3K/AKT pathway is involved in PTX-induced cytoplasmic accumulation of transfected p21, we determined the phosphorylation status of p21 using an antibody specific for the AKT phosphorylation consensus motif. As shown in Figure 4a (upper panel), this specific antibody binds p21 immunoprecipitated from PTX-treated cells but not from control cells.

We next considered the possibility that the phosphorylation of p21 might cause the cytoplasmic sequestration of p21 by promoting its association with 14-3-3, scaffold protein that specifically binds phosphoserine containing proteins. Figure 4a (lower panel) shows that in RPMI-2650 cell treated with 10nM PTX, the association between p21 and 14-3-3 was increased.

LY 294002 is a specific inhibitor of PI-3 kinase that activates AKT. We tested the effect of LY 294002 by adding it to RPMI-2650 cells that were transiently transfected to express HA-p21. As illustrated in Figure 4b, treatment with LY 294002 reversed the ability of PTX to decrease the nuclear accumulation of p21. In addition, in cells cotransfected with HA-p21 and a dead kinase form of AKT which acts as a dominant-negative AKT mutant (Zugasti *et al*., 2001), PTX failed to induce cytoplasmic accumulation of p21 (Figure 4b). However, we found that forced expression of a constitutive active form of AKT induced cytoplasmic localization of transfected p21, indicating that AKT reproduced the action of PTX on RPMI-2650 cells (Figure 4b).

We then determined the effect of PTX on AKT activity. This activity was first assessed by measuring its kinase activity on histone H2B. As shown in Figure 4c (left panel), a basal level of AKT activity was detected in RPMI-2650 cells and this level was unchanged after addition of 10 nM

Figure 3 PTX induces cytoplasmic accumulation of p21. (**a**) Subcellular distribution of endogenous and ectopic-expressed p21 in control or paclitaxel-treated cells. RPMI-2650 cells were transiently transfected with a pcDNA3 vector allowing the expression of HA-epitope-tagged version of p21. Control and PTX-treated cells (10 nM) were suspended in hypotonic buffer, homogenized and separated in a nuclear pellet fraction and a supernatant fraction. The fractions were analysed by Western blot with anti-p21, anti-HA, anti-p53, anti-lamin B1 and anti-actin antibodies. A protein, corresponding to a histone molecular weight, was observed after Ponceau's staining, only in the nuclear fraction. (**b**) RPMI-2650 cells were transiently transfected with a pcDNA3 vector allowing the expression of HA-epitope-tagged version of p21. Cells were treated or not with PTX, 10 nM for 24 h, then fixed and stained with anti-HA antibody and DAPI. (**c**) Quantification of the percentage of cells expressing HA-p21 and displaying nuclear staining (black bar) or nuclear and cytoplasmic staining (white bar). Results represent the mean ± s.d. of three independent experiments

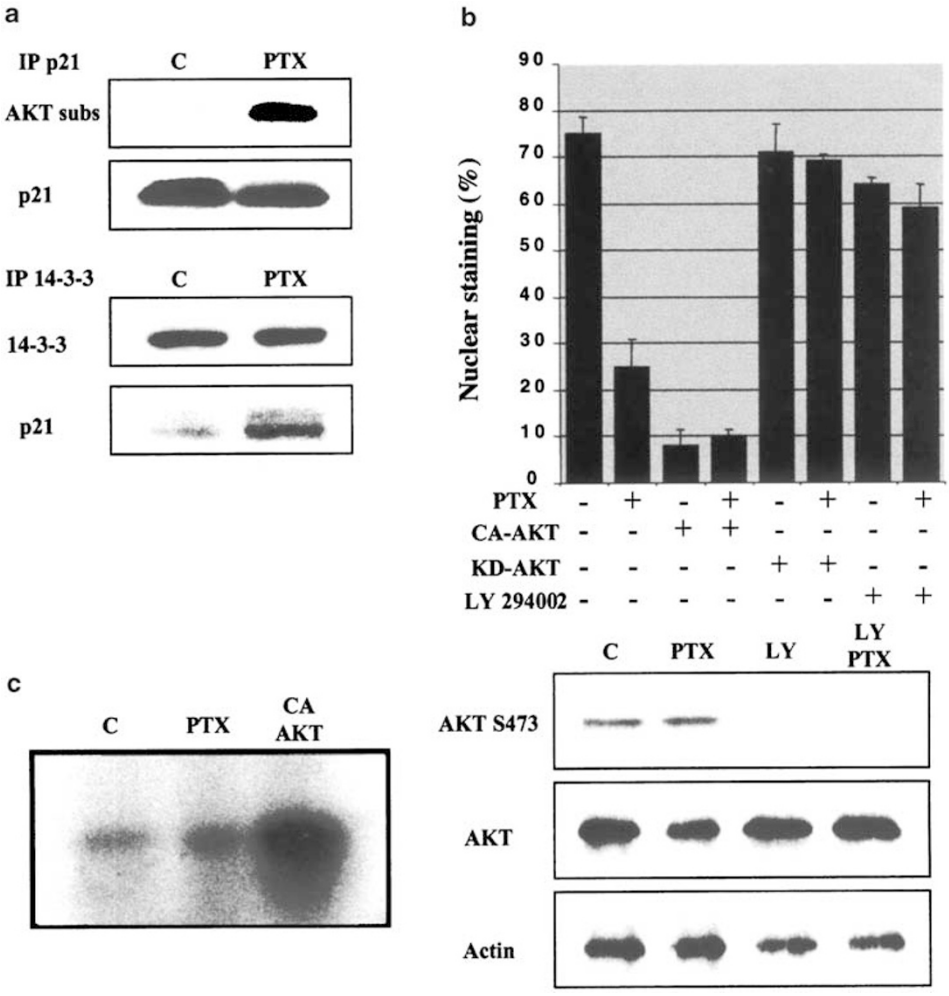


Figure 4 AKT-dependent phosphorylation of p21 is required for cytoplasmic accumulation of p21. (**a**) PTX (10 nM, 24 h) induces accumulation of phosphorylated form of p21. After transfection of RPMI-2650 cells with a vector allowing the expression of p21, p21 (upper panel) or 14-3-3 were immunoprecipitated. Phosphorylated p21 form was detected by immunoblot analysis using a phospho-specific antibody against the AKT consensus sequence (upper panel). Lower panel, immunoblot analysis of p21. (**b**) RPMI-2650 cells were transiently transfected with vectors allowing the expression of HA-epitope-tagged version of p21 and either a constitutive active form (CA-AKT) or a dead kinase form of AKT (KD-AKT). Transfected cells were treated or not with PTX, 10 nM, 24 h, in the presence or absence of LY 294002 (20 μM), fixed and stained with anti-HA antibody and DAPI. The percentage of cells with a p21 nuclear staining was determined on three independent experiments. (**c**) Comparable AKT activity was observed in control and PTX-treated cells. RPMI-2650 cells were transiently transfected with vectors allowing the expression of AKT or a constitutive active form of AKT (CA-AKT). Immunoprecipitated AKT was incubated with histone H2B as substrate. A representative autoradiograph is shown (left panel). AKT activity in control and PTX (10 nM)-treated cells in the presence or absence of LY 294002 (20 μM) was also assessed by Western blot analysis using an antibody specific for its active Ser473 phosphorylated form of AKT (right panel)

PTX. The activity of the transfected constitutive active form of AKT was used as a positive control (Figure 4c, left panel). AKT activity was also measured by Western blot analysis using an anti-phospho-AKT antibody directed against Ser473 (Figure 4c, right panel). Here again, PTX was unable to modify AKT activity. Furthermore, inhibition of the PI-3 kinase pathway with LY 294002 reduced AKT activation in both control and PTX-treated cells (Figure 4c, right panel). Taken together these data indicate that although AKT activity is required for cytoplasmic accumulation of p21 in PTX-treated cells, its activity is not directly regulated by PTX.

PTX-induced cytoplasmic accumulation of transfected p21 results in an inhibition of its inhibitory effect on cell cycle progression

In order to demonstrate that accumulation of p21 in the cytoplasm induced by low doses of PTX results in an inhibition of its function, we determined whether the cellular response to transfected p21 was affected by its accumulation in cytoplasm. As previously shown (Cayrol *et al*., 1998), we found that transfection of p21 resulted in a decrease in the proportion of cells in S phase and an accumulation of cells with a G2/M DNA content compared to controls (Figure 5a). Treatment of

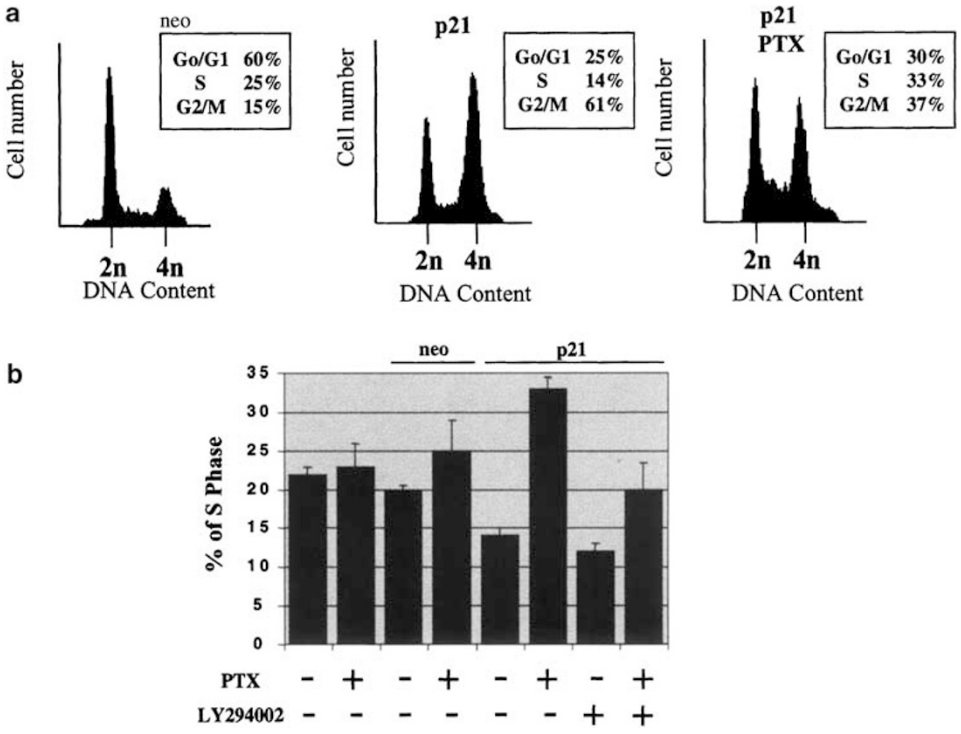


Figure 5 PTX reversed cell cycle effect of transfected p21. (**a**) Cell cycle analysis of RPMI-2650 cells transfected with pcDNA3/neo, pcDNA3/p21 in the presence and absence of PTX (10 nM, 24 h). Cells were fixed and labelled with propidium iodide. (**b**) Quantitative determination of proportion of cells in S phase. Cells transfected with pcDNA3/neo or pcDNA3/p21 were treated or not with 10 nM PTX for 24 h in the presence or absence of LY 294002; cells were fixed and labeled with propidium iodide. Results represent mean ± s.d of three independent experiments

cells with 10 nM PTX for 24 h reversed p21 cell cycle effects. Indeed, we observed an increase in the proportion of cells in the S phase and a decrease in the accumulation of cells in G2/M phases of the cell cycle (Figure 5a). A quantification of S phase (Figure 5b) and G2/M cell repartition (data not shown) indicate that the addition of PTX to p21-transfected cells reversed the inhibitory effect of p21 in correlation with p21 cytoplasmic accumulation. LY 294002 inhibited the action of PTX on both cytoplasmic accumulation of p21 (Figure 3b) and increase in proportion of cells in S phase (Figure 5b). Similar results were obtained on the G2/M proportion. These findings indicate that the PI-3 kinase/AKT pathway is involved in the ability of PTX to reverse the influence of overexpression of p21 on the cell cycle.

Discussion

The experiments described here show that in cancer cells, a dose of PTX that is not high enough to activate the spindle assembly checkpoint, induces the p53-dependent pathway p21 expression and its accumulation in the cytoplasm, effectively inhibiting its antiproliferative action.

p21 mediates the p53-dependent checkpoint pathway that restrains cells with a 4N DNA content from entering S phase after aberrant mitosis induced by microtubule acting agents (Khan and Wahl, 1998; Lanni and Jacks, 1998; Stewart *et al*., 1999a) or to any late mitotic failure rendering a cell tetraploid (Andreassen *et al*., 2001). However, in accord with previous results (Giannakakou *et al*., 2001; Torres and Horwitz, 1998), we show here that low doses of PTX, unable to activate the spindle assembly checkpoint, induce a p53-dependent expression of p21. The question arises on the role of this p21 expression. It has been recently shown that in some breast carcinoma cell lines, microtubule-depolymerizing agents, nocodazole and vincristine, cause a p21-mediated G1 and G2 arrests before cells reach mitosis, but this p21 induction is p53-independent and only observed after high doses of microtubule-destabilizing agents (Blajeski *et al*., 2002). A G1 and a G2 arrests resulting in a complete inhibition of A549 and MCF-7 cell proliferation have been associated with the ability of low doses of PTX to upregulate p21 (Giannakakou *et al*., 2001). In contrast to this, in spite of the increase in p21 in RPMI-2650, we failed to observe a significant arrest in the G1 and G2 phases of the cell cycle and an inhibition of cell proliferation. In order to avoid PTX cell accumulation (Jordan *et al*., 1996), RPMI-2650 cells were treated with low doses of PTX for 24 h, and cell number was counted 3 days later. We have checked firstly, that in these experimental conditions, proliferation of A549 cells was not inhibited although p21 was upregulated (data not shown) and

secondly, that a continuous 3 days treatment with low doses of PTX resulted in an inhibition of RPMI-2650 cell proliferation. Taken together, these data indicate that inhibition of A549 and MCF-7 cell proliferation observed in the presence of low doses of PTX (Giannakakou *et al*., 2001) is rather the result of PTX accumulation than a consequence of p21 induction.

The inhibition of cell growth stems from a nuclear action of p21 (el-Deiry *et al*., 1994). We showed that microtubule damage induced by low doses of PTX triggers an accumulation of p21 in the cytoplasm, whereas p53 remains in the nucleus. AKT phosphorylates p21 at Thr145, and it has been recently shown that p21 phosphorylation abrogates the inhibitory effect of p21 on cell proliferation (Rossig *et al*., 2001; Zhou *et al*., 2001). Different molecular mechanisms are thought to be involved in such actions. Phosphorylation of p21 by AKT has been demonstrated to induce the release of PCNA from complexes with p21 and to regulate the activities of the CDK2 and CDK4 complexes (Rossig *et al*., 2001), whereas it triggers a relocalization of p21 in the cytoplasm (Zhou *et al*., 2001). It has also been shown that phosphorylation of p21 by AKT enhances protein stability (Li *et al*., 2001). Our results demonstrated that the PI-3 kinase/AKT-dependent pathway is involved in the cytoplasmic accumulation of either endogenous or transfected p21, triggered by low doses of PTX. For instance, we found that: (i) PTX increased levels of the AKT phosphorylated form of p21, (ii) transfection of a constitutive active form of AKT reproduced the action of PTX on cytoplasmic accumulation of transfected p21, and (iii) inhibition of the PI3K/AKT pathway by either LY 294002 (a specific inhibitor of PI-3 kinase) or transfection of a dead form of the AKT kinase inhibited the ability of PTX to trigger cytoplasmic accumulation of p21. 14-3-3 binding was known to result in cytoplasmic accumulation of cell cycle regulators such as Cdc25, WEE1 and CDK2 (Li *et al*., 2001). Our results indicate that low doses of PTX promote the association between 14-3-3 and p21 suggesting a relation between p21 phosphorylation, its association with 14-3-3 and its cytoplasmic retention. However, the molecular mechanism responsible for the increase in AKT-dependent p21 phosphorylation in PTX-treated cells remains to be elucidated. So far, we have been unable to demonstrate that paclitaxel regulates the PI-3 kinase/AKT pathway directly. Protein phosphorylation is a dynamic balance of the activities of protein kinases and phosphatases. The hypothesis that low doses of PTX regulate p21 phosphatase activity is now under investigation.

The interaction between p21 and 14-3-3 may be important for retaining the phosphorylated form of p21 in the cytoplasm and thereby inhibiting its antiproliferative activity. Indeed, we found that blockade of cell cycle progression of cancer cells in G1 and G2 phases after p21 transfection was reversed by low doses of PTX. PTX induces a G1/S cell cycle checkpoint in nontransformed cells, whereas transformed cells were unable to arrest in G1 after treatment with PTX (Trielli *et al*., 1996). We hypothesize that a constitutive activation of the PI-3 kinase/AKT pathway leading to p21 phosphorylation in cancer cells enables them to escape the p21-mediated cell cycle checkpoints. A cytoprotective effect of p21 against PTX-induced cytotoxicity has been reported (Barboule *et al*., 1997; Yu *et al*., 1998; Li *et al*., 1999; Stewart *et al*., 1999b). It was assumed that inhibition of cyclin B/CDK1 kinase was involved in this action of p21 either by causing cell cycle exit from mitosis (Barboule *et al*., 1997; Yu *et al*., 1998) or by preventing entry into mitosis (Blagosklonny *et al*., 2000). This apoptotic pathway requires cell cycle block in mitosis and is consequently activated by higher concentrations of PTX than those required for p21 induction. However, it has been shown that PTX-mediated cell death may occur by a different mechanism. Whereas at high PTX concentrations apoptosis results from a terminal mitotic arrest, at low concentrations cell death occurs in the absence of mitotic arrest after an aberrant mitosis (Torres and Horwitz, 1998; Chen and Horwitz, 2002). The possibility that cytoplasmic accumulation of p21 is involved in the escape to the apoptotic pathway independently of mitotic arrest is now under investigation. In support to this hypothesis, it has been shown that a cytoplasmic association of 21 with the kinase ASK1 is involved in the protection against apoptosis during monocytes differentiation (Asada *et al*., 1999). Our data, together with previous results (Barboule *et al*., 1997; Yu *et al*., 1998; Stewart *et al*., 1999b; Schmidt *et al*., 2000), suggest that p21 is critical for cell response to microtubule damage. Cytoplasmic accumulation of p21 in cancer cells treated with low doses of PTX allows cancer cell to escape to its antiproliferative effect and may increase their survival.

Materials and methods

Cell culture

The human nasal squamous carcinoma RPMI-2650 cell line was obtained from the American Type Culture Collection and was grown in Eagle's minimum essential medium with Earle (Sigma) supplemented with 10% fetal calf serum, 2 mM glutamine, 0.1 mg/ml penicillin/streptomycin and 1 mM pyruvate (Sigma). RPMI-2650 cells were cultured in humidified 5% CO_2/95% air at 37°C.

Chemical and antibodies

PTX was purchased from Bristol Myers and dissolved as a 10^{-4} M solution in Me_2SO. LY 294002 was obtained from Sigma. Polyclonal p21 (C-19) antibody was purchased from Santa-Cruz. Monoclonal p53 antibody (DO-1) and polyclonal antibody to CDK2 were both obtained from Pharmingen. Polyclonal anti-AKT, phospho-AKT (S473) and antiphospho-(Ser/Thr) AKT substrate were purchased from cell signaling. Polyclonal antiphospho-histone-H1 and anti-14-3-3 β C-20 were, respectively, from Euromedex and Santa-Cruz. Monoclonal anti-HA (12CA5) was from Roche. Monoclonal antibodies against actin, lamin B1 and α tubulin were, respectively, from Chemicon Int., Serotec and Amersham. pcDNA3 HA-AKT, pcDNA3-HA were provided by Dr T Franke. pCMV-neo and pCMV-E6 were provided by Dr B Vogelstein.

Cell transfection

Cells were transfected with the calcium phosphate method. Cells were seeded, and treated overnight with a mix containing DNA, $CaCl_2$ (125 mM) and transfection buffer (BES 25 mM pH 6.95, NaCl 140 mM, Na_2HPO_4 0.75 mM) when almost plated. The calcium phosphate precipitate was then washed with PBS.

Cell proliferation

Cells were plated in Petri dishes and treated with various concentrations of PTX. After 24 h, cells were washed with PBS and new medium was added without PTX. Cells were grown for 4 days, then scraped with trypsin and counted with a cell counter (Coulter Z2).

Cytometry

Cells were centrifuged at 1000 *g* for 8 min, the pellet was washed with PBS and recentrifuged for 5 min at 1000 *g*. Pelleted cells were fixed in 80% ethanol overnight at −20°C. The samples were centrifuged, washed with PBS and resuspended in 10 mg/ml of propidium iodide (Sigma) and 0.1% RNAse A (Sigma) in PBS. After a 15 min incubation at 37°C, the distribution of cells in the different phases of the cell cycle was determined by analytical flow cytometry using FACSscan software (Beckton Dickinson).

Subcellular fractionation

Cells (2×10^7) were washed with ice-cold PBS and resuspended in 1 ml of Buffer A (HEPES 20 mM, KC1 10 mM, $MgCl_2$ 10 mM, PMSF 17 μg/ml, aprotinin 8 μg/ml, leupeptin 2 μg/ml, pespstatin 1 μg/ml, TPCK 100 μg/ml and TLCK 50 μg/ml). After homogenization (20–30 strokes with a Dounce B-type pestle homogenizer) and checking that >99% cells were lysed, samples were centrifuged at 1000 *g* for 10 min at 4°C to pellet nuclei. The nuclear pellet was resuspended in Buffer B (10 mM Tris pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$ and 0.1% NP 40). It was then laid down on a sucrose cushion (30% sucrose, Tris HCl 50 mM pH 8.3, $MgCl_2$ 5 mM, EDTA 0.1 mM) and centrifuged at 1100 *g* for 10 min at 4°C. The resulting nuclear pellet was resupended in Buffer C (Tris 50 mM pH 7.5, NaCl 250 mM, EDTA 5 mM, Deoxycholate 0.25%, SDS 0.1%, Triton 0.1% and protease inhibitors). The supernatant, resulting from the initial low-speed centrifugation, was used as cytoplasmic fraction.

Immunofluorescence staining

Cells were grown on glass coverslips, washed once with PBS and fixed with PBS/3.7%. formaldehyde for 15 min at 4°C. Cells were permeabilized in PBS/0.25% Triton X-100 for 5 min at room temperature. Nonspecific antibody sites were blocked with a PBS/1% PCS solution at room temperature for 30 min. Primary antibodies were diluted in PBS/1% serum for 1 h at room temperature for anti-p21 (C-19) and anti-tubulin and at 37°C for anti-HA. Secondary antibodies coupled with Texas-red or FITC were incubated for 1 h at room temperature. DNA was stained by incubating the cells with a 0.1 mg/ml solution of DAPI in PBS. Coverslips were then mounted on glass slides with Mowiol (DAKO).

Western blotting, immunoprecipitation and AKT kinase activity

Cells were washed with PBS and lysed in ice-cold lysis buffer (Tris-HCl pH 7.5, 250 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.1% Triton, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 100 μg/ml PMSF and TPCK, 50 μg/ml TLCK, 2 μg/ml leupeptin, 1 μg/ml pepstatin and 1 μg/ml aprotinin). Lysates were cleared by centrifugation. For Western blot analysis, 100 μg of proteins were resolved by SDS–PAGE electrophoresis and transferred on to nitrocellulose membranes by semidry blotting. The membranes were hybridized with antibodies. For equal loading control, the blot was probed with actin antibody. For immunoprecipitation, rabbit polyclonal CDK2 or p21 antibodies were added to 1 mg of precleared cell lysates and incubated at 4°C for 2 h on a rotative platform. Immune complexes were recovered with protein A sepharose (Pharmacia) and washed three times with lysis buffer. Immune complexes were separated on 12.5% SDS–PAGE and revealed with rabbit polyclonal CDK2 or p21 antibodies. AKT kinase activity was measured on cells transfected with HA epitope-tagged forms of either AKT or its constitutively activated form. HA monoclonal antibody was added to 500 μg of protein extracted. Immune complexes were washed with kinase assay buffer (Tris 25 mM pH 7.5, $MgC1_2$ 10 mM, $MnCl_2$ 10 mM, DTT 2 mM, β glycerophosphate 5 mM, orthovanadate 0.1 mM, ATP 50 μM) and incubated for 30 min at 30°C in the presence of 1 μg of Histone H2B and 2 μCi of (^{32}P)γ-ATP. The reaction was stopped by boiling the samples in Laemmli SDS samples buffer for 5 min and samples were resolved by 12.5% SDS–PAGE. The gels were dried and subjected to autoradiography.

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