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Evidence that low doses of Taxol enhance the functional transactivatory properties of p53 on p21 waf promoter in MCF-7 breast cancer cells

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Abstract In the present study, we evidence how in breast cancer cells low doses of Taxol for 18 h determined the upregulation of p53 and p21 waf expression concomitantly with a decrease of the anti-apoptotic Bcl-2. P53 and its gene product, the mdm2 protein, in treated cells exhibits a prevalent nuclear compartmentalization, thus potentiating p53 transactivatory properties. Indeed, the most important finding of this study consists with the evidence that Taxol at lower concentrations is able to produce the activation of p21 promoter via p53. Prolonged exposure of MCF-7 cells to Taxol (48 h) resulted in an increased co-association between p21 and PCNA compared to control and this well fits with the simultaneous block of cell cycle into the G2/M phase.

Keywords: Taxol; p21 waf; p53; Breast cancer cells

1. Introduction

Taxol is a chemotherapic drug specifically effective against prostate, ovarian, breast and lung cancer. Its primary mechanism of action is related to the ability to stabilize the microtubules and to disrupt their dynamic equilibrium [1–6].

Treatment of cells with Taxol interferes with the normal reorganization of the microtubule network, and inhibits the formation of normal spindle at metaphase required for mitosis and cell proliferation. These effects lead to an arrest of the cells in the G2/M phase of the cell cycle and eventually to apoptotic cell death [7–9].

The biological responses to Taxol may vary depending on cell type and drug concentration.

An aspect extremely intriguing rises from the evidence that low doses of Taxol in human lung cancer, though still unable to alter all microtubule network, upregulate p53 and its nuclear compartmentalization [10]. Indeed, the polymerization of microtubules with the extension of their minus end, induced by Taxol, may facilitate the translocation of p53, using dynein as carrier, from the cytosol into the nucleus [10,11].

In this compartment, p53 stimulates the expression of proteins involved in a wide network of signals that act through two major apoptotic pathways: the extrinsic death receptor signalling which triggers caspases activation and Bid cleavage, and the intrinsic mitochondrial pathway, which shifts the balance in the Bcl-2 family towards the pro-apoptotic members, enhancing mitochondrial permeabilization with consequent release of cytochrome *c* and direct caspases activation [12]. These events have been previously reported in human neuroblastoma, ovarian and breast carcinoma cells that underwent Taxol treatment [13–15].

Taxol-initiated apoptosis has been also associated with increase of p21 waf/Cip protein, a key regulator of cell cycle and DNA synthesis, which expression is regulated by p53-dependent and/or independent mechanisms [16–19].

P21 binds to various cyclin–CDK complexes and inhibits their activity, thus resulting in a block in cell cycle [20]. An alternative mechanism through which p21 inhibits cell cycle progression lays on its capability to recruit PCNA, then enabling this factor to interact with the DNA polymerase δ and ε activities [21,22].

In the present study, we have explored if low doses of Taxol "per se" are able to enhance the transactivatory properties of p53 producing the upregulation of p53-classically dependent gene, such as p21 waf, involved in the regulation of cell apoptosis and in the progression of cell cycle.

2. Materials and methods

2.1. Materials

DMEM/Ham's F-12, L-glutamine, penicillin/streptomycin, calf serum (CS), bovine serum albumin, aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate and Taxol were purchased from Sigma (Milan, Italy). FuGENE 6 was from Roche

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Applied Science (Milan, Italy). Dual luciferase kit and TK Renilla luciferase plasmid were provided by Promega (Madison, WI). [γ^{32}P]-ATP and ECL system come from Amersham Biosciences.

The plasmid WWP-Luc containing human p21 waf promoter (2.4 kb) was kindly given by Dr. Wafik El-Deiry (Howard Hughes Medical Institute, Philadelphia); pCMV-wt p53 plasmid, pCMV-p53 plasmid mutant and pCMV empty vector were generously provided by Dr. G. Daniel (Department of Health and Human Service, Natl. Inst. Env. Health Sci., Res. Triangle Park, NC). Thin layer chromatography (TLC) aluminium sheets were from MERK (Milan, Italy).

2.2. Cell lines and culture conditions

Human breast cancer MCF-7 cell line was cultured in DMEM/Ham's F12 (1:1) medium supplemented with 5% CS, 1% L-glutamine and 1% penicillin/streptomycin. The cells were cultured in phenol-red-free DMEM (PRF-SFM-DMEM) containing 0.5% bovine serum albumin, 1% L-glutamine and 1% penicillin/streptomycin, 24 h before each experiment. Next, the 70% confluent cells, synchronized in PRF-SFM-DMEM (day 0) [23] were treated with different doses of Taxol (2, 6, 12, 50, 100 nM) for 18 and 48 h.

2.3. Cell viability

The viability of the cells was assessed by morphological analysis using trypan blue exclusion assay. Cells in the exponential growth phase were plated and grown overnight; then, the medium was changed and shifted for 24 h with PRF-SFM-DMEM. At the end of this incubation the cells were exposed for 18 and 48 h to different concentrations of Taxol, as reported in Fig. 1. Cells were trypsinized and incubated in a 0.5% trypan blue solution for 10 min at room temperature and viable numbers were determined microscopically by counting trypan blue negative cells in a counting chamber (Burker, Brand, Germany).

2.4. Transfections and luciferase assay

MCF-7 cells were seeded (1×10^5 cells/well) in DMEM/F12 supplemented with 5% CS in 24-well plates. Cells were co-transfected with the plasmid WWP-Luc containing human p21 waf promoter and pCMV-empty vector or pCMV-p53 mutant plasmid or pCMV-wt p53 plasmid, in SFM using FuGENE6 according to the manufacture's instructions with a mixture containing 0.1 μg/well of each specific plasmid and 25 ng/well of TK Renilla luciferase plasmid. 24 h after the transfection the medium was changed and the cells were treated in PRF-SFM-DMEM in the presence of 2, 6 and 12 nM of Taxol for 18 h. The firefly and Renilla luciferase activities were measured by using a dual luciferase kit. The firefly luciferase data for each sample were normalized on the basis of the transfection efficiency measured by Renilla luciferase activity.

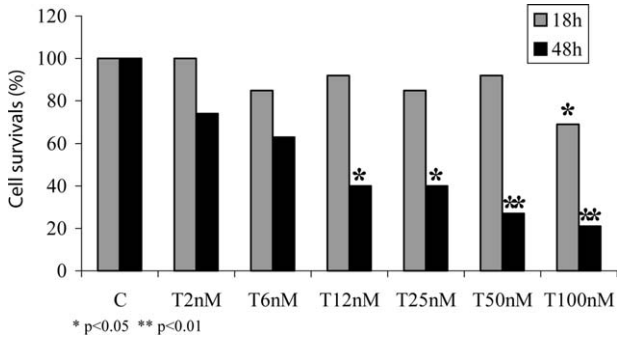

Fig. 1. Cell viability of MCF-7 cells after Taxol treatment. MCF-7 cells were plated in six-well plates at a density of 2×10^5 cells/well and grown 24 h to be completely attached to the surface of the plates. The day after, the medium was switched to serum-free medium for 24 h. Next, the cells were added of different doses of Taxol ranging from 2 nM until 100 nM and incubated for 18 and 48 h. Values are the average of four triplicate independent experiments, and are expressed as percentage of the controls, determined by standardizing untreated cells to 100%. $^{*}P < 0.05$ $^{**}P < 0.01$ as compared to untreated cells. The S.D. was lower than 0.25%.

2.5. Immunoprecipitation and Western blotting

MCF-7 cells were grown in 100 mm dishes to 70–80% confluence, shifted to SFM for 24 h and lysed. Cytoplasmic protein lysates were obtained with a buffer containing 50 mM HEPES, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 10 mM EGTA, pH 7.5, 10% glycerol, 1% Triton X-100 and protease inhibitors (2 μM Na_3VO_4, 1% PMSF, 20 μg/ml aprotinin). Following the collection of cytoplasmic proteins, the nuclei were lysed with the buffer containing 20 mM KOH–HEPES, pH 8, 0.1 mM EDTA, 5 mM $MgCl_2$, 0.5 M NaCl, 20% glycerol, 1% Np-40 and inhibitors (as above) [24].

The association of PCNA (Proliferating Cell Nuclear antigen) and p21 waf/Cip and/or phospho p21 (Thr 145) proteins was assessed by immunoprecipitation (IP) and Western blotting (WB) in 500 μg protein lysates using appropriate antibodies (as specified in the figure legends), while the association of dynein and p53 proteins was determined by immunoprecipitating the nuclear fractions with anti-dynein antibody and then blotting for anti-p53 antibody and anti-β-tubulin. In IPs, protein lysates were incubated in HNTG buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol and 0.2 mM Na_3VO_4) at 4 °C for 4 h with the primary antibodies, and then agarose beads conjugated with Protein A/G Agarose (Sigma) were added for another 1 h. The immunoprecipitated proteins were washed three times with the HNTG buffer and separated by SDS–PAGE (polyacrylamide gel electrophoresis).

The expression of different proteins was tested by WB in 50 μg of protein lysates or in 500 μg of immunoprecipitated cell proteins using an anti-p21 WAF, anti-phospho-p21 waf (Thr 145), anti-PCNA, anti-p53, anti-mdm2, anti β-actin, anti-p85, anti-Lamin B, anti-GAP-DH, anti-β-tubulin and anti-dynein pAbs from Santa Cruz Biotechnology (Heidelberg, Germany), anti-phospho-Akt (Ser 473), anti phospho p-Bcl-2 (Ser 70), anti-pBcl-2 and anti-caspase-9 pAbs from Cell Signaling Technology (Beverly, MA, USA).

Proteins were transferred to a nitrocellulose membrane, probed with primary antibody and then stripped and reprobed with the appropriate antibodies. The antigen–antibody complex was detected by incubation of the membranes for 1 h at room temperature with a peroxidase-coupled anti-IgG antibody and revealed using the ECL system. Blots were then exposed to film and bands of interest were quantified by densitometer (Mod 620 BioRad). The results obtained were expressed in term of arbitrary densitometric units.

2.6. Immunofluorescent microscopy

50% confluent cultures, grown on coverslips, were shifted to SFM for 24 h and then treated either for 18 or 48 h with 2, 6 or 50 nM of Taxol. Cells were then fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed three times with PBS, and incubated for 1 h with a mixture of primary Abs recognizing p53 and p21. The anti-p53 monoclonal Ab (mAb) (Santa Cruz) at 2 mg/ml was used for p53 staining; anti p21 polyclonal Ab (pAb) (Santa Cruz) at 2 mg/ml was used to detect p21. Following the incubation with primary Abs, the slides were washed three times with PBS, and incubated with a mixture of two secondary Abs, each 1 mg/ml concentrated. A rhodamine-conjugated donkey anti-mouse IgG (Calbiochem) was used as a secondary Ab for p53 and a fluorescein-conjugated donkey anti-rabbit IgG (Calbiochem) was used for p21. The cellular localization of p53 and p21 was studied with confocal microscope with 1000× magnification. The optical sections were taken at the central plane.

2.7. FACS analysis

Serum-starved cells for 24 h were given Taxol for 18 and 48 h at the doses reported in the figures. After this incubation cells were trypsinized, washed with PBS and fixed for 1 h in ice-cold 70% ethanol. The samples were then washed once with PBS and resuspended in 1 ml of staining solution (10 mg/ml RNasi A, 10 mg/ml propidium iodide in PBS). The samples were then incubated at room temperature in the dark for at least 30 min. FACS analysis was performed using CellFeet software (Becton Dickinson, NJ). At least 2×10^4 cells/sample were measured.

2.8. PI-3 kinase activity

PI-3K activity associated with p85, was assessed by standard protocol provided by the manufacturer of the p85 antibody (Upstate Biotechnology). Briefly, p85 was IP from 500 μg of protein lysate with an anti-p85 p-Ab, the negative control was performed using a cell lysate where p110 catalyzing subunit of PI3K, was previously removed by pre-

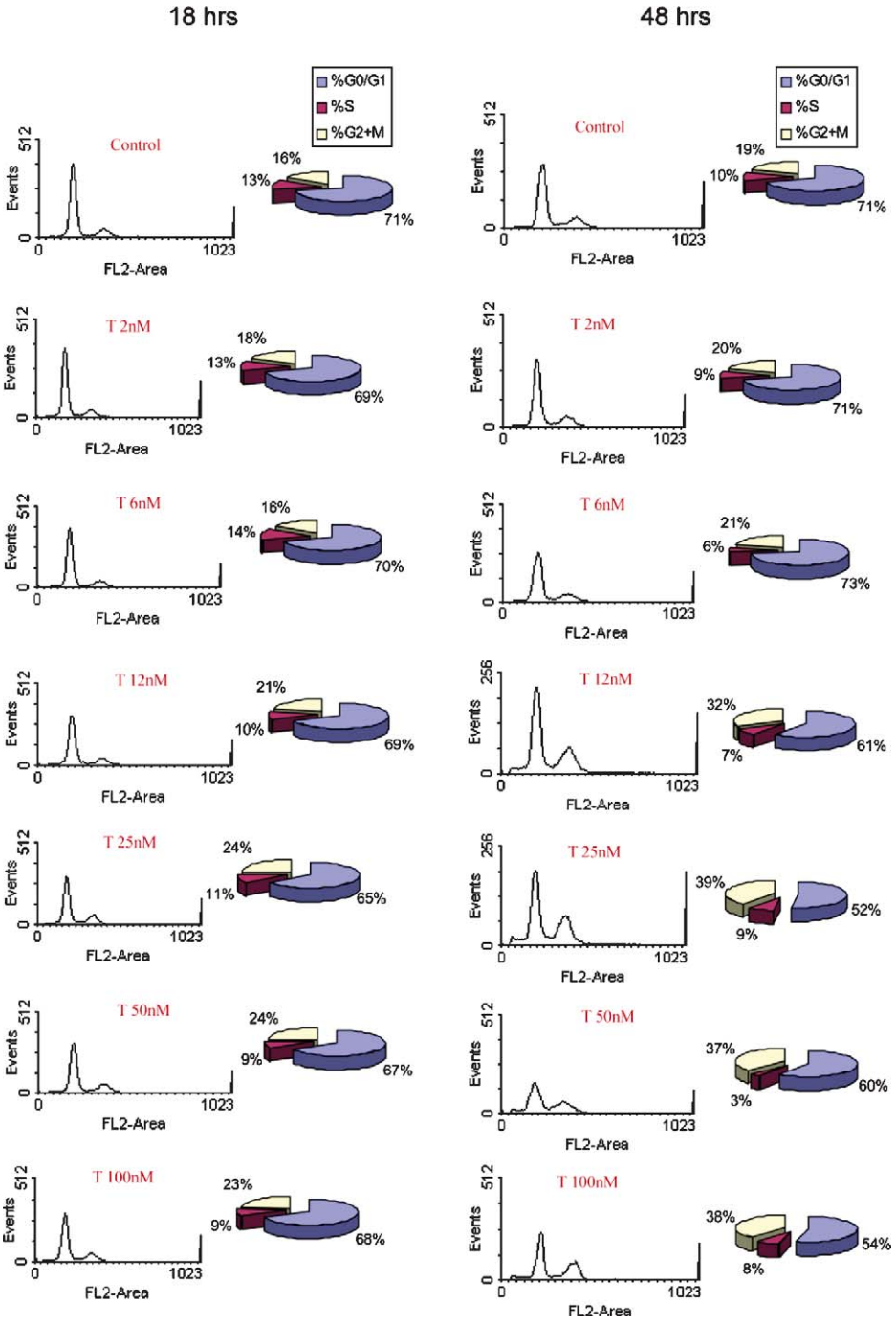

Fig. 2. Effect of different doses of Taxol on cell cycle progression of MCF-7 breast cancer cells. Serum-starved MCF-7 cells for 24 h were incubated for 18 and 48 h with the different concentrations of Taxol as shown in figure. Then the cells were collected and stained with propidium iodide (see Section 2) to be analyzed by FACS. DNA histograms were measured using Cell Fit software and the percentage of G0/G1, S and G2/M cells were calculated. Data are representative of four independent experiments.

incubation with the respective antibody (1 h at room temperature) and subsequently immunoprecipitated with Protein A/G-agarose. As a positive control, MCF-7 were treated with 100 nM insulin for 24 h before lysis and immunoprecipitated with anti-p85 from 500 μg of cell lysates. The immunoprecipitates were washed once with cold PBS, twice with 0.5 M LiCl, 0.1 M Tris (pH 7.4) and finally with 10 mM Tris, 100 mM NaCl, 1 mM EDTA. The presence of PI3K activity in immunoprecipitates was determined by incubating the beads with reaction buffer containing 10 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 50 μM ATP, 20 μCi [γ^{32}-P] ATP, and 10 μg of L-α-phosphatidylinositol-4,5-bis phosphate (PI-4,5-P_2) for 20 min at 37 °C. The reactions were stopped by adding 100 μl of 1 M HCl. Phospholipids were extracted with 200 μl of $CHCl_3$/methanol. For extraction of lipids, 200 μl of chloroform:methanol (1:1, v/v) were added to the samples and vortexed for 20 s. Phase separation was facilitated by centrifugation at 5000 rpm for 2 min in a tabletop centrifuge. The upper phase was removed, and the lower chloroform phase was washed once more with clear upper phase. The washed chloroform phase was dried under a stream of nitrogen gas and redissolved in 30 μl of chloroform. The labelled products of the kinase reaction, the PI phosphates, then were spotted onto *trans*-1,2-diaminocyclohexane–*N*,*N*,*N*′,*N*′-tetraacetic acid-treated silica gel 60 TLC plates. Radioactive spots were visualized by autoradiography.

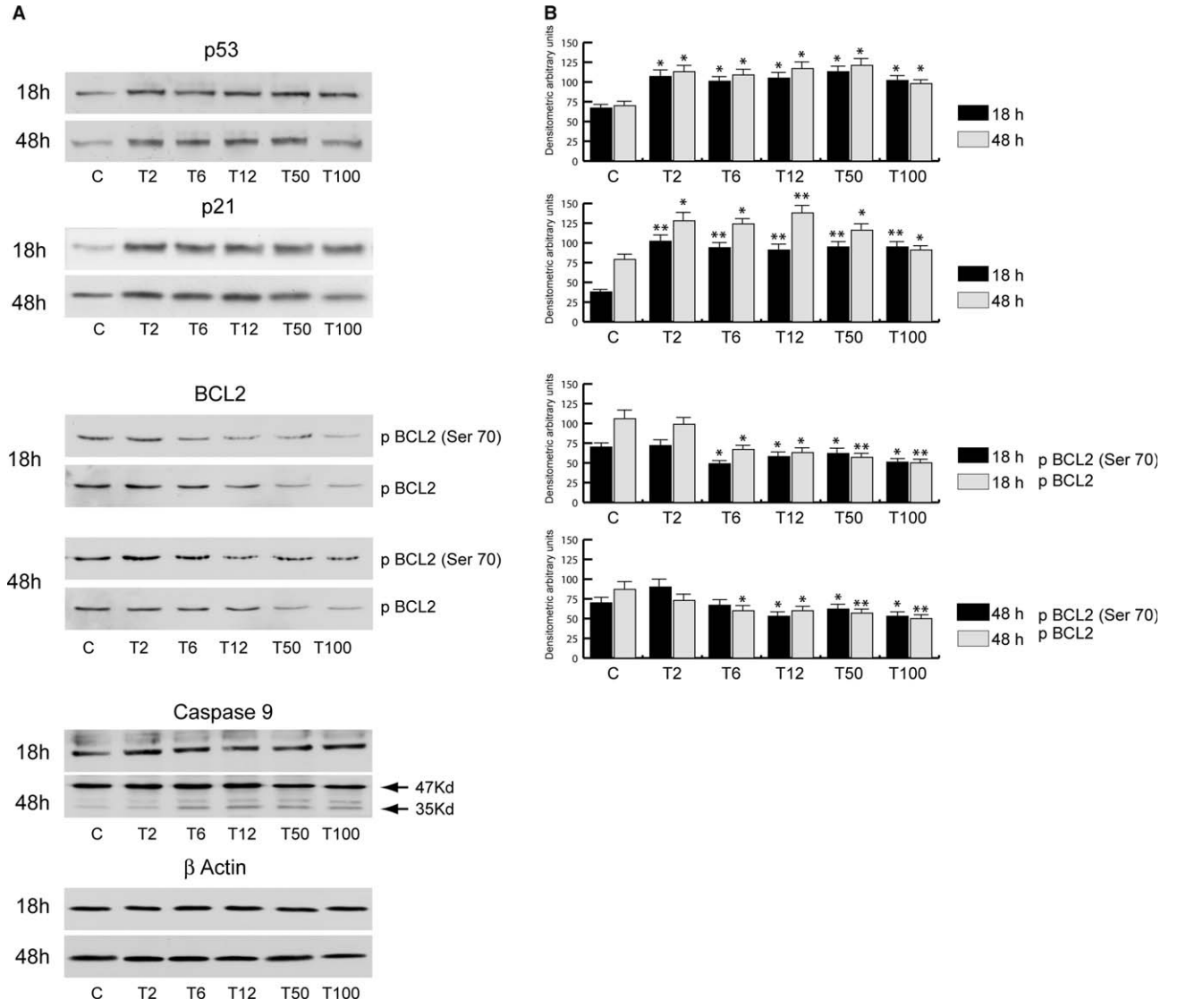

Fig. 3. Low doses of Taxol affect p53 expression and its target proteins p21 WAF and Bcl-2. (A) MCF-7 cells underwent Taxol treatment (from 2 to 100 nM) for 18 and 48 h were harvested and lysed to detect p53 protein expression in 50 μg of total cell lysates. The same filter was stripped and reprobed with anti-p21 waf, anti-Bcl-2, anti caspase-9 and anti-β-actin antibodies. C: control; T: Taxol at different nM concentrations. β-Actin serves as a loading control. Representative results are shown. (B) The histograms represent the mean ± S.D. of three separate experiments in which bands intensity for p53, p21 and Bcl-2 were evaluated in term of arbitrary densitometric units. $^{*}P < 0.05$; $^{**}P < 0.01$ vs C.

2.9. DNA ladder formation

The ladder assay is based on the oligonucleosomal DNA fragmentation of nuclear DNA that can be visualized by ethidium bromide staining following electrophoresis. MCF-7 cells were plated in 100-mm dishes (1×10^6 cells/dish); serum-starved cells were treated with Taxol (2, 6, 50 and 100 nM) for 48 h.

At the end of the incubation period cells were trypsinized and combined with floating cells in the same culture. DNA was isolated by lysing the cells in 400 μl of 0.2% Triton-X, 20 mM EDTA, and 10 mM Tris, pH 8.0, for 20 min on ice. The DNA fragments were harvested by centrifugation for 20 min at 12000 rpm. After the addition of 400 μl of phenol–chloroform the supernatant was centrifuged at 12000 rpm for 5 min and then was precipitated with sodium acetate (400 μl) and ethanol (800 μl) for 24 h at −20 °C.

Afterwards the supernatant was centrifuged at 12000 rpm for 20 min, dried and incubated for 1 h at room temperature with a buffer containing 500 μg/ml RNase A. The DNA fragments were resolved by electrophoresis at 75 V on 1% agarose gel impregnated with ethidium bromide, detected by UV transillumination, and photographed.

2.10. Statistical analysis

Each data point represents the mean ± S.D. of at least three experiments. The data were analyzed by analysis of variance using the STATPAC computer program.

3. Results

3.1. Taxol decreases basal growth rate of MCF-7 cells in a dose/time-related manner

High doses of Taxol (100 nM) since 18 h of treatment inhibited MCF-7 cells proliferation. The same inhibitor effects were produced by lower doses of Taxol starting from 12 nM after 48 h of incubation (Fig. 1). These results well correlates with FACS analysis demonstrating a block of MCF-7 cell cycle into the G2/M phase after 48 h of drug treatment at the doses ranging from 12 to 100 nM (Fig. 2).

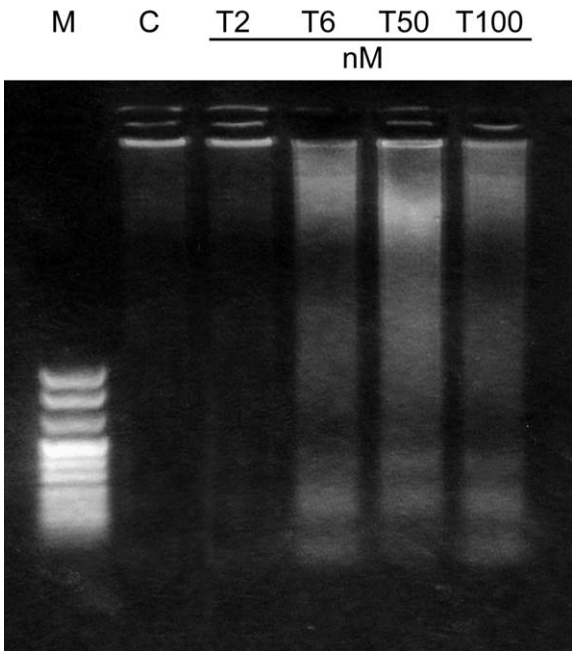

Fig. 4. Treatment of MCF-7 cells with Taxol induces DNA fragmentation. DNA gel electrophoresis of Taxol-treated (T) (lanes 2–5) and control (C) (lane 1) MCF-7 cells. DNA from MCF7 cell treated for 48 h with 2, 6, 50 and 100 nM of Taxol was extracted as detailed in Section 2, then separated in 1.5% agarose gel electrophoresis. DNA from a 48 h control (untreated) culture was prepared in the same way. M, Marker.

3.2. Cell survival pathway is affected by taxol

To investigate if Taxol "per se" might produce early changes in cell apoptotic and/or survival signals, first of all we focused our attention on the effects of the drug on p53 and p21 waf expression.

It is worth to mention that low doses of Taxol incubated for 18 and 48 h are able to enhance both p53 and p21 waf protein expressions, while the anti-apoptotic Bcl-2, in its total content and consequently in its phosphorylative status (phospho-Bcl Ser70) resulted downregulated (Fig. 3 A and B). However, it is note worthy to observe how the relative phospho-Bcl-2 levels tend to be enhanced by Taxol treatment since the decrease of Bcl-2 protein content occurs much faster than its phosphorylation. Indeed, the ratio of densitometric values of the bands between phospho-Bcl-2 /Bcl-2 protein varied from 0.6 in control sample, to 1.2 in treated sample. Drug treatment triggers cell apoptotic events only after 48 h, eliciting the cleavage of caspase-9, which is detectable from Taxol 6 nM and persists at the higher doses (Fig. 3A). This was also confirmed by agarose DNA electrophoresis that demonstrates the formation of the characteristic ladder of DNA at 6, 50 and 100 nM of Taxol (48 h), while it is absent at lower concentration of the drug as well as in the control sample (Fig. 4).

On the basis of the reported findings, even in the presence of low doses of Taxol, p53 may potentiate its transactivatory properties on the regulatory region of target gene through its faster translocation into the nucleus. To prove this we studied p53 nuclear/cytosolic compartmentalization in MCF-7 cells in the presence of low and high doses of the drug incubated for both 18 and 48 h.

3.3. Taxol induced P53 and P21 intracellular relocation

WB analysis performed, respectively, in the nuclear and cytosolic cell lysates showed that p53 and its gene product, the mdm2 protein, after 18 h of drug treatment, exhibit a prevalent nuclear compartmentalization, while p21 waf protein

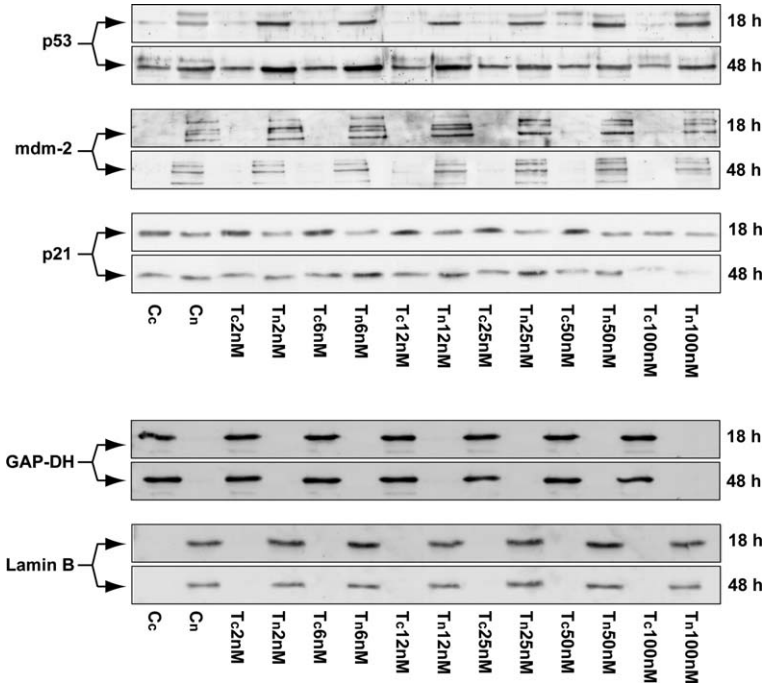

Fig. 5. Different subcellular compartmentalization of p53, mdm2 and p21 waf in cells treated with Taxol. MCF-7 cells treated with Taxol, at the concentrations reported in the figure, were harvested and cytosolic (c) and nuclear (n) protein lysates were subject to WB with an anti-p53, anti-mdm2, anti-p21 antibodies. C, control; T, Taxol-treated cells. The expressions of a nuclear protein, Lamin B, and a cytoplasmatic enzyme, GAP-DH, were assessed by stripping and reprobing the filters to verify the purity of fractions.

appears mostly localized in the cytosol (Fig. 5). Drug treatment of MCF-7 cells for 48 h increases p53 levels in both nuclear and cytosolic compartments until 25 nM of cell exposure to Taxol. At the same time, p21 waf protein tends to diffuse from the cytosol into the nucleus (Fig. 5). P53 uses dynein to translocate into the nucleus since it co-immunoprecipitates with the microtubule-motor protein and with β-tubulin (Fig. 6). In the end, we observed that the amount of protein bound to dynein in the nuclear compartment is increased upon Taxol treatment at 48 h.

The subcellular localization of both p53 and p21 proteins was also investigated by confocal microscopy using immunofluorescent staining of p53 (rhodamine-conjugated antibody, red staining) and p21 (fluorescein-conjugated antibody, green staining) proteins.

At 18 h of drug treatment, p53 tended to accumulate much more into the nucleus (a very strong nuclear staining was ob-

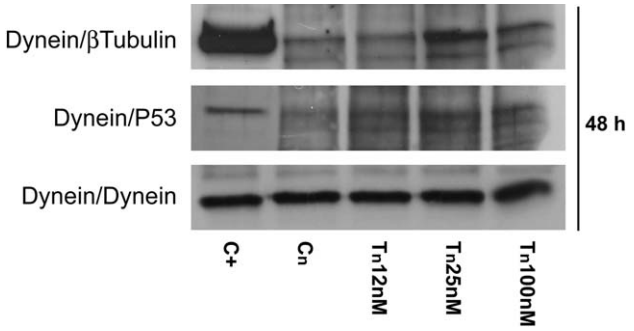

Fig. 6. P53 co-immunoprecipitates with dynein. MCF-7 cells treated with Taxol (12, 25, 100 nM) for 48 h were harvested and nuclear (n) protein lysates were subject to IP experiment by using an anti-dynein antibody/protein A/G complex followed by WB with anti-β-tubulin antibody; anti-p53 antibody; anti-dynein antibody. C+, 50 μg of soluble cell protein; Cn, nuclear control.

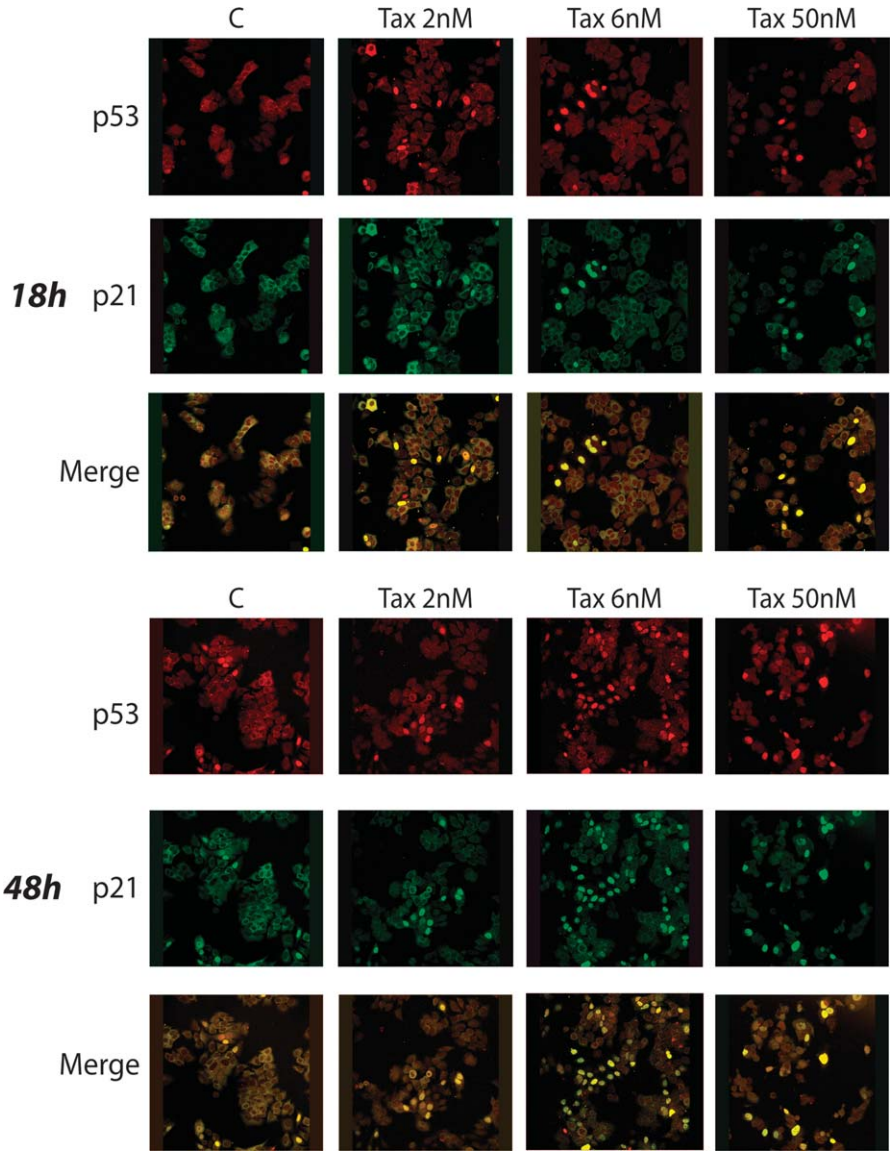

Fig. 7. Immunolocalization of p53 and p21 WAF in MCF-7 cells treated with Taxol. MCF-7 cells were treated with Taxol, at the indicated doses, for 18 and 48 h and subsequently fixed and stained with a rhodamine-conjugated donkey anti-mouse IgG as secondary Ab for p53 (red), or with a fluorescein-conjugated donkey anti-rabbit IgG for p21 (green). Staining was analysed by confocal laser-scanning microscopy. Co-localization of p53 and p21 waf is visible as yellow staining generated where the color images merge. Images are optical sections at intervals of 0.3 μm along the *z*-axis from the bottom of the cell to the top of the nucleus.

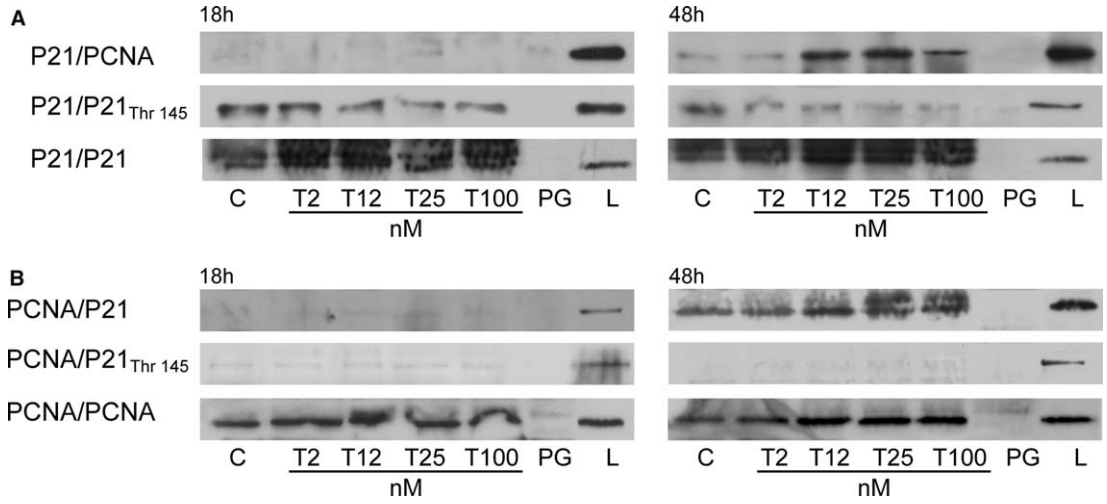

Fig. 8. Expression of p21-PCNA complex in MCF-7 cells treated with different doses of Taxol. MCF-7 cells treated with Taxol at the indicated concentrations (nM) for both 18 and 48 h were lysed and immunoprecipitated in (A) with: an anti-p21 waf antibody/protein A/G complex followed by WB with specific anti-PCNA antibody (upper panel), with anti-p21 (Thr 145) antibody (middle panel) and with anti-p21 antibody (lower panel); in B with: an anti PCNA antibody/protein A/G complex followed by WB with specific anti-p21 antibody (upper panel), with anti-p21 (Thr 145) antibody (middle panel) and with anti-PCNA antibody (lower panel). C, control; L, 50 μg of soluble cell protein not subjected to IP; PG, 0.5 mg of soluble cell protein immunoprecipitated only with protein A/G-agarose beads; T, Taxol treated cells. Results are representative of three independent experiments.

served in about 10% of cells treated with 2 and 6 nM Taxol, and in 30% of cells treated with 50 nM Taxol) respect to control sample, where, instead, a quite uniform localization in both nuclear and cytoplasmatic compartments is showed (Fig. 7). At the same time, in untreated cells, p21 resulted to be prevalently retained in the cytosol and following Taxol treatment it tends to translocate in the nucleus.

For instance, at 48 h of incubation, while 5% of control cells showed a strong nuclear co-localization of p53 and p21, in cells treated with 2 and 6 nM of Taxol the percentage increased to 15% and 50%, respectively. Nevertheless, it is worth to mention that the detectable cell compartmentalization of p53 and p21 waf proteins, in the presence of high concentrations of Taxol (50 nM), are in all likelihood not reliable since morphological changes turning cell polygonal shape into rounding ones occur and produces loss of adhesion (Fig. 7).

The cytosolic retention of p21 waf at 18 h, probably makes it unable to interact with nuclear proteins like PCNA and to inhibit cell cycle.

To determine if the level of co-association between p21 and PCNA in MCF-7 treated cells may correlate with the inhibition of cell cycle progression we performed, at both 18 and 48 h, two sets of IP experiments by using whole-cell lysates incubated:

(i) with anti-p21 waf antibody followed by immunoblotting with an anti-PCNA antibody (Fig. 8A); and
(ii) with anti-PCNA antibody followed by immunoblotting with an anti-p21 antibody (Fig. 8B).

As shown in Fig. 8A, PCNA protein was present in immunoprecipitates from MCF-7 cells treated for 48 h with 12, 25 and 100 nM of Taxol. The highest levels of co-IP of p21/PCNA were concomitant with the alteration of cell cycle.

The same filters obtained at 18 and 48 h of drug treatment were reprobed with an anti-phospho-p21 waf (Thr 145) antibody, that represents the phosphorylated form of the protein unable to inhibit cell cycle. In accordance, the results demonstrated that in untreated and treated MCF-7 cells the phospho-p21 waf (Thr 145) is present at 18 h and it slightly decreases under Taxol (Fig. 8A). On the other hand, Taxol treatment prolonged up to 48 h significantly reduced the level of phosphorylated p21 waf protein compared with that obtained at 18 h. To asses that p21 is immunoprecipitated in all experimental conditions we reprobed the same blots with an anti-p21 antibody.

In the reverse IP experiment (IP with anti-PCNA antibody and blot with anti-p21 antibody) (Fig. 8B) the results confirm that PCNA and p21 waf associated only following 48 h drug treatment, while phospho-p21 (Thr 145) is not present in the IPs both at 18 and 48 h. The same filters were then blotted for anti-PCNA antibody to verify PCNA protein in immunoprecipitates.

3.4. Evidence that low doses of taxol are able to potentiate transactivatory properties of P53

53 nuclear compartmentalization, induced by low doses of Taxol, leads us to assume that the treatment with the drug per se may potentiate the functional transactivating properties of this protein.

PTo support this assumption we treated MCF-7 transfected with a p21 promoter conjugated with a luciferase reporter gene. Our results show, for the first time, how low doses of Taxol are able to induce the activation of p21 promoter (Fig. 9). This activation was drastically potentiated by the ectopic overexpression of wild-type p53 and abrogated in the presence of p53 mutant construct.

3.5. Effect of low doses of taxol on PI3K/Akt signal

When we evaluated the effect of the drug on the PI3K/Akt pathway, which is crucial in maintaining cell survival signal, we observed that Taxol (25 and 100 nM) for 18 h induced an early activation of PI3K and consequently this leads to an increase of phospho-Akt levels (Fig. 10A and B). Such

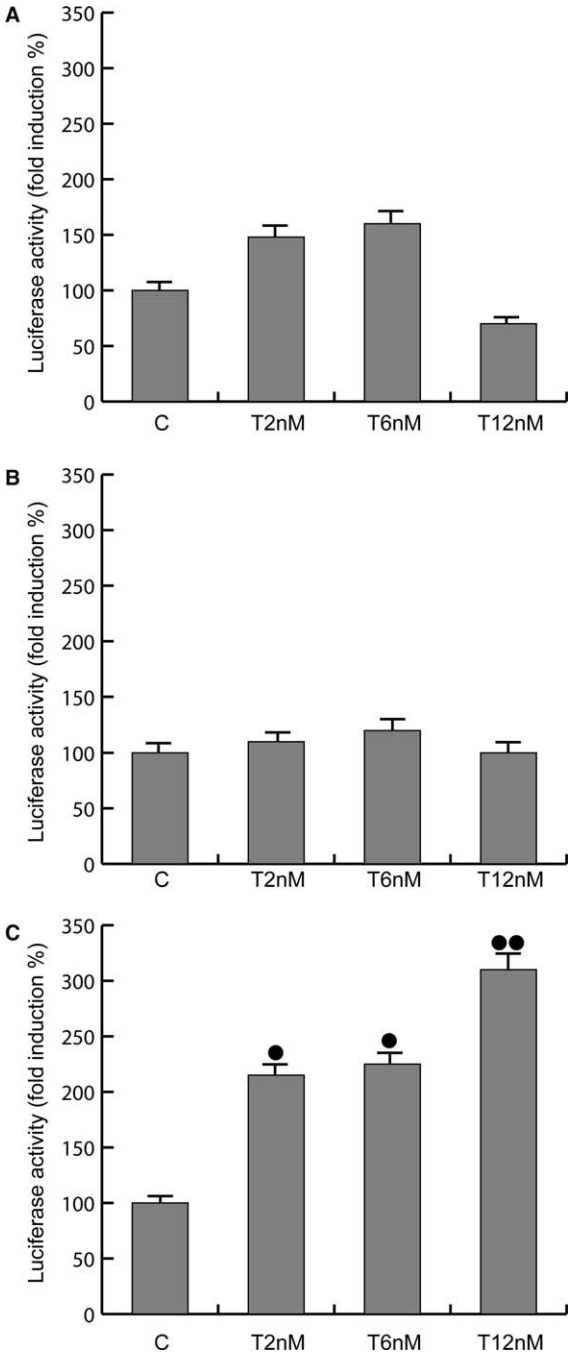

Fig. 9. Taxol induces p21 WAF promoter activity via p53. MCF-7 cells were co-transfected with the p21 waf promoter-luciferase construct and pCMV empty vector (A), pCMV-p53 mutant (B) and pCMV-p53 wild-type (C). After 8 h of transfection cells were treated with low doses of Taxol (T): 2, 6 and 12 nM for 18 h. MCF-7 cells were lysed and luciferase activities were measured using the dual luciferase reporter assay system as described under Section 2. Each column/bar represents the relative p21 waf promoter (luciferase) activity and is the mean ± S.D. of three independent experiments in triplicate. Levels of significance vs control (C): $^{\bullet}P < 0.05$; $^{\bullet\bullet}P < 0.01$.

effect is not longer noticeable at 48 h of drug exposure when G2/M cell population, analyzed in flow cytometry, appears to increase significantly together with a concomitant down regulation of the anti-apoptotic signal: Bcl-2 protein, as previously documented.

4. Discussion

Taxol has been shown to be effective as an anticancer agent in a variety of tumoral cell types [8,25–27].

As a result of cell exposure to Taxol, events characteristic to cell cycle arrest and apoptosis might take place. These effects seem to be linked to the different concentration of the drug as well as to the tissues and/or cell line used.

In the present study we demonstrate how low doses of Taxol have some apparent conflictory effects: an enhanced p53 expression involved in the regulation of programmed cell death on one hand, and the activation of PI3 kinase involved in cell survival on the other. The apparent contradictory effects have been previously reported by other authors in the same breast cancer cell type where the rapid enhancement of PI3K/Akt together with an increased of survivin expression, occurs only to counteract the increased p53 expression [28]. Indeed, it is well known how p53 might inhibit PI3K/Akt pathway by double mechanisms: first through the PTEN activation, a specific inhibitor of PI3K activity, as well as through an enhanced Akt degradation via proteosoma [29,30]. However, the functional effect of the enhanced PI3K/Akt signal, induced by low doses of Taxol, seems to be vanished by the decrease of the anti-apoptotic Bcl-2, which in such a way, fails to have any protective role in controlling mitochondrial permeability and then to inhibit the release of cytochrome *c*. Indeed, in the same circumstance, low doses of Taxol (6 nM) incubated for 48 h in MCF-7 cells are able to induce the cleavage of caspase-9 and the formation of DNA ladder. These apoptotic events precede the block of cell cycle, which occurs as from 12 nM of Taxol.

The reduced levels of Bcl-2, linked to the enhanced p53 expression under Taxol treatment, counteract the activation of PI3K/Akt pathway. P53 protein, in turn, through its binding to the negative response *cis*-elements of the Bcl-2 gene promoter may transcriptionally downregulate the expression of the anti-apoptotic protein [31].

Here, we observed that in the presence of low doses of Taxol, p53 co-immunoprecipitates with dynein, a microtubule-motor protein that requires ATP to move along microtubules with their cargoes.

Our data, together with previous results, suggest that in such circumstances p53 may use dynein to translocate into the nucleus, an event which is potentiated by Taxol treatment [10,11]. Besides, an other protein that comes down in a p53/dynein complex is the β-tubulin prevalently present in treated cells. Thus, the effect of the polymerizing agent on microtubule assemblement, consistent with their end elongation, might functionally contribute to p53 nuclear translocation where the p53-transactivatory properties would appear enhanced. The deep drop of Bcl-2 expression and the enhancement of p21 waf protein may stem from this.

Indeed, the most important finding of the present study is consistent with the evidence that Taxol, at lower concentrations, is able to transactivate p21 promoter resulting in the enhancing of p21 protein expression. This response is linked to p53 wild-type expression in MCF-7 cells, since the presence of a p53 mutant abrogates the transactivation of p21 promoter under Taxol treatment. In the same vein, the nuclear localization of p53 induced by low doses of Taxol after 18 h is followed by the enhancement of its gene product: the mdm2 protein, which shows the same sub-cellular distribution reported for p53.

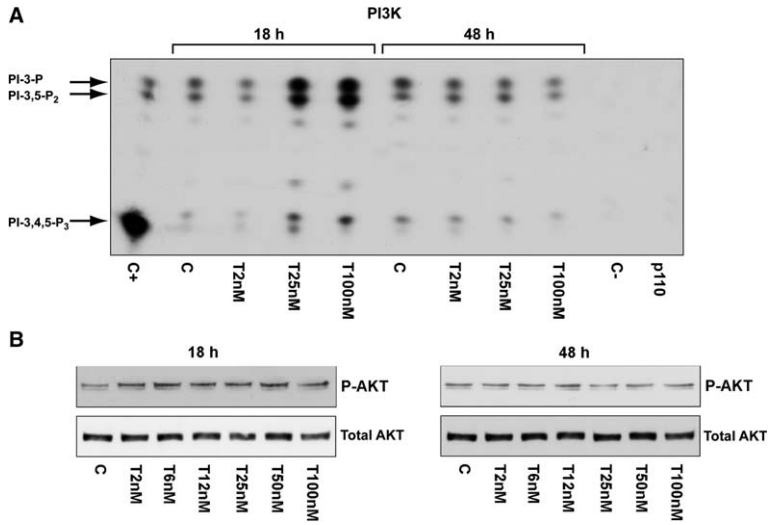

Fig. 10. Transitory Taxol activation of PI3 kinase pathway. (A) MCF-7 cells treated with Taxol (at the indicated doses) for 18 and 48 h were lysed and immunoprecipitated with an anti-p-85 antibody. The immunocomplexes were assayed for their ability to phosphorylate PI to PIP using [γ^{32}P]-ATP at 37 °C for 20 min. The PIP was resolved by TLC and autoradiographed. As a positive control (C+) MCF-7 cells were treated with 100 nM of insulin for 24 h. The negative control (p110) was performed using MCF-7 lysate, where p110, the catalyzing subunit of PI3K, was previously removed by preincubation with the respective antibody (1 h at room temperature) and subsequently immunoprecipitated with protein A/G-agarose. PI-3,4,5-P3: phosphatidylinositol 3,4,5-triphosphate; PI-3,5-P2: phosphatidylinositol 3,5-diphosphate; PI-3-P: phosphatidylinositol 3-phosphate. An additional negative control (C−) is represented by MCF-7 cells previously treated with worthmanin for 30 min. Untreated cells: (C), Taxol-treated cells: (T). The autoradiographs presented are representative of experiments that were performed at least three times with repetitive results. (B) WB of phospho-Akt (Ser 473) levels from MCF-7 cells treated with the indicated doses of Taxol (T) at 18 and 48 h of incubation. Total Akt levels are showed as a loading control. Results are representative of three independent experiments.

It is worth to observe that in this scenario MCF-7 cell cycle is not substantially modified, given that p21 waf result mostly stored in the cytosol and this could prevent its binding with nuclear factors such as PCNA [32–34]. Indeed, at the same time, the enhanced activation of phospho-Akt, as here reported, may serve to recruit p21 protein into the cytosol, where it is unable to act as an inhibitor of CDKs and thereby to inhibit cell cycle progression [16,33,34]. Results from this study showed that prolonged treatment of MCF-7 cells with Taxol up to 12 nM induced an evident co-association between p21 protein and PCNA which was concomitant with the decay of the phosphorylative status of p21 waf (Thr 145) together with the arrest of cell cycle. These findings supported the evidence that when phospho-Akt is not activated due to the prolonged treatment with Taxol, p21, mainly present in the ipo-phosphorylated form, is able to co-immunoprecipitate with PCNA. The latter event well correlates with the block of cell cycle into the G2/M phase that progressively increases with the elapsing of time of the drug exposure as well as with the dose of Taxol used.

In conclusion, in the present study we have demonstrated how low doses of Taxol, without affecting cell cycle, may induce the enhanced expression of p53 protein and its prevalent nuclear translocation with well featured apoptotic events occurring in breast cancer cells. This, furthermore, supports the potential benefit of the association of low doses of Taxol with the classic chemotherapic agents. The combined treatment may potentiate the effect of low doses of chemotherapics reducing thus the harmful systemic effects mostly occurring during the treatment of breast carcinomas.

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