Cisplatinum and Taxol Induce Different Patterns of p53 Phosphorylation

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Abstract

Posttranslational modifications of p53 induced by two widely used anticancer agents, cisplatinum (DDP) and taxol were investigated in two human cancer cell lines. Although both drugs were able to induce phosphorylation at serine 20 (Ser20), only DDP treatment induced p53 phosphorylation at serine 15 (Ser15). Moreover, both drug treatments were able to increase p53 levels and consequently the transcription of *waf1* and *mdm-2* genes, although DDP treatment resulted in a stronger *inducer* of both genes. Using two ataxia telangiectasia mutated (ATM) cell lines, the role of ATM in drug-induced p53 phosphorylations was investigated. No differences in drug-induced p53 phosphorylation could be observed, indicating that ATM is not the kinase involved in these phosphorylation events. In addition, inhibition of DNA-dependent protein kinase activity by wortmannin did not abolish p53 phosphorylation at Ser15 and Ser20, again indicating that DNA-PK is unlikely to be the kinase involved. After both taxol and DDP treatments, an activation of hCHK2 was found and this is likely to be responsible for phosphorylation at Ser20. In contrast, only DDP was able to activate ATR, which is the candidate kinase for phosphorylation of Ser15 by this drug. This data clearly suggests that differential mechanisms are involved in phosphorylation and activation of p53 depending on the drug type.
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Introduction

The product of the tumor suppressor gene p53 plays a crucial role in determining cellular response to various stress conditions [1–4]. In cancer cells, p53 has been reported to modulate the cytotoxicity of some drugs either by increasing or decreasing their activity. Tumor cell lines expressing wild-type (wt) p53 were reported to be more sensitive to *cis*-dichloro-diammine platinum (DDP) and other anticancer drugs and less sensitive to tubulin interactive drugs such as taxanes than cells not expressing p53 or expressing mutated p53 [5,6]. The results of the screening program at the NCI on 60 cell lines reported that taxol had a tendency to show higher activity in cells not expressing p53 compared to those expressing p53 [7]. Opposite results were obtained with DDP [7], although recently an increased DDP cytotoxicity was found to be associated with loss of p53 function [8].

Taxol and DDP are two widely used anticancer agents that have different mechanisms of action; DDP reacts with DNA inducing inter-, intrastrand crosslinks [9], whereas taxol binds to tubulin, causing an inhibition of its depolymerization [10]. However, both compounds are able to activate p53 in different cell systems to a comparable extent [11–13]. For both drugs, it has been shown that increased p53 levels are able to activate at least one of the downstream targets of p53, the *WAF1* gene encoding for p21 [11,13].

The pathway that leads to p53 activation after damage is complex and many proteins have been shown to be involved in this process [14–16]. It appears that different kinds of damage, particularly at the DNA level, use different "sensors" to obtain p53 activation [17–19]. The proteins reported to play a role in signalling mechanisms resulting in p53 activation following DNA damage include DNA-dependent protein kinase (DNA-PK), ataxia telangiectasia mutated (ATM), ATM-Rad3 related (ATR), hCHK1, hCHK2, and histone acetyl transferases [19–30]. Whereas the former protein kinases have been shown to specifically phosphorylate p53 at its amino or carboxy terminus, the latter histone acetyl transferases have been demonstrated to acetylate the p53 carboxy terminus [26,27]. Recent evidence suggests that after DNA damage, both the amino and carboxy terminus of p53 can be modified by phosphorylations and acetylations [17,18,24–26] and that these posttranslation modifications seem to be specifically associated with the kind of damage produced. Moreover, it appears that these posttranslational modifications of p53
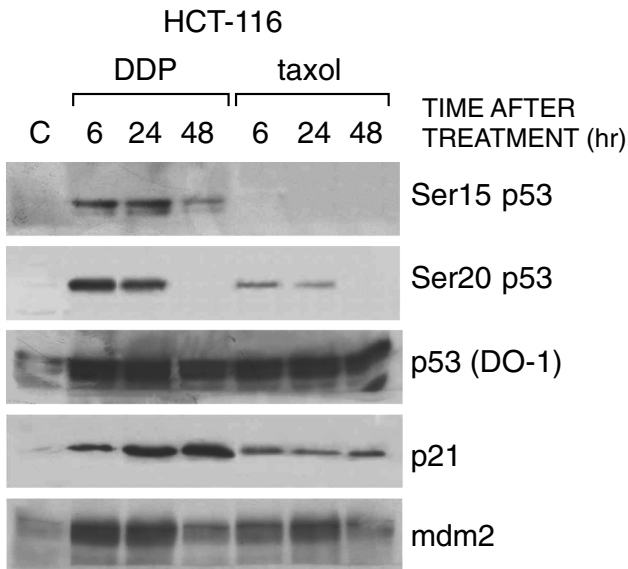
HCT-116

Figure 1. *Western blot analysis in HCT-116 cells treated with DDP or taxol. Extracts were obtained at different times after treatment. Blots were hybridized with antibodies recognizing p53 phosphorylated at Ser15, Ser20, p53 (DO-1), p21, and mdm-2.*

could represent a crucial step in determining the specificity of downstream gene activation and thus be an important determinant in the decision between p53-induced cell cycle arrest or apoptosis.

Because DDP and taxol are very active anticancer drugs with different mechanisms of action, it was of interest to investigate if activation of different kinases induced different posttranslational modifications of p53. In this article, taxol and DDP induced accumulation of p53 in human ovarian cancer cells and different patterns of p53 phosphorylation are reported. The ability of p53 to bind DNA and to activate downstream genes has also been evaluated.

Materials and Methods

Cells and Drugs

The human ovarian cancer cell line A2780 was grown in RPMI1640 supplemented with 10% FCS. The human colocarcinoma cell line HCT-116 was maintained in Iscove's modified Dulbecco medium, supplemented with 10% FCS. The Epstein–Barr virus transformed lymphoblastoid cell lines IARC1663 (normal cell line), AT11 and AT13 (AT homozygote cell lines) were kindly provided by Dr. J. Hill (IARC, Lyon, France) and cultured in RPMI 1640 medium supplemented with 20% heat-inactivated FCS. DDP and taxol (Bristol-Myers Squibb, Syracuse, NY, USA) were dissolved in medium immediately before use; acetyl-Leu-Leu-norleucinal (LLnL) (Sigma, Milan, Italy) was dissolved just before use. Wortmannin (Sigma, Milan, Italy) was stored in DMSO at −20°C and diluted in medium just before use. DDP and taxol concentration inhibiting the growth by 50% (IC50) in the two different cell lines were determined by growth inhibition assay (data not shown).

Western Blotting Analysis

Cell extracts were prepared by lysing cells in 50 mM Tris–HCl pH 7.4, 250 mM NaCl, 0.1% Nonidet np-40, 5 mM EDTA, 50 mM NaF in the presence of aprotinin, leupeptine, and PMSF as proteases inhibitors, for 30 minutes on ice. Insoluble material was pelleted at 13,000×g for 10 minutes at 4°C and the protein concentration was determined using a Biorad assay kit (BioRad, Milan, Italy). Forty micrograms of total cellular proteins was separated on SDS-PAGE and electrotransferred to nitrocellulose. Immunoblotting was carried out with anti-p53 monoclonal antibodies (DO-1, Santa Cruz Biotechnology, Santa Cruz, CA), anti-p21, and anti–DNA-PK polyclonal antibodies (Santa Cruz Biotechnology), anti–mdm-2 and anti-ATR polyclonal antibodies (Calbiochem, Milan, Italy), and the polyclonal anti-hCHK2 antibody kindly provided by Dr. M. Foiani (University of Milan, Italy). Antibody binding was revealed by peroxidase secondary antibodies and visualised using enhanced chemiluminescence (ECL) (Amersham, Milan, Italy).

Immunoblot Analysis of p53 Phosphorylation

Antibodies specifically recognizing phosphorylated p53 at Ser15 and Ser20 were generated as previously described [20]. Different replicates of blots obtained as described above were incubated with these specific antibodies and the presence of phosphorylated p53 revealed with the ECL (Amersham). The blots were re-probed with anti-p53 antibodies (DO-1) to verify the presence of p53 protein.

Nucleic Extracts and EMSA

Cells (10^6) were lysed in ice in buffer containing 10 mM Hepes pH7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, and 0.8% Nonidet NP-40. Nuclei were pelleted, extracted for 1 hour in extraction buffer (20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF), and cleared by centrifugation at 12,000×g for 15 minutes. Ten micrograms of nucleic extracts was incubated on ice for 1 hour in 15 µl of buffer containing 20 mM Hepes pH 7.5, 100 mM NaCl, 1 mM
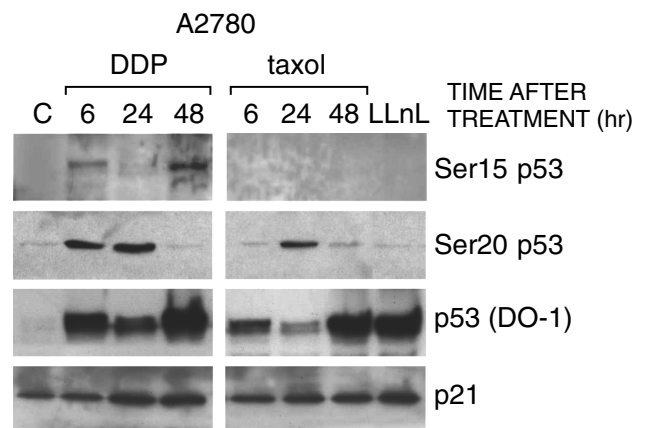
A2780

Figure 2. *Western blot analysis in A2780 cells treated with DDP or taxol. Experimental conditions were as reported in Figure 1. Cells were also treated with 50 µM of LLnL for 3 hours and cellular extracts taken at the end of treatment.*
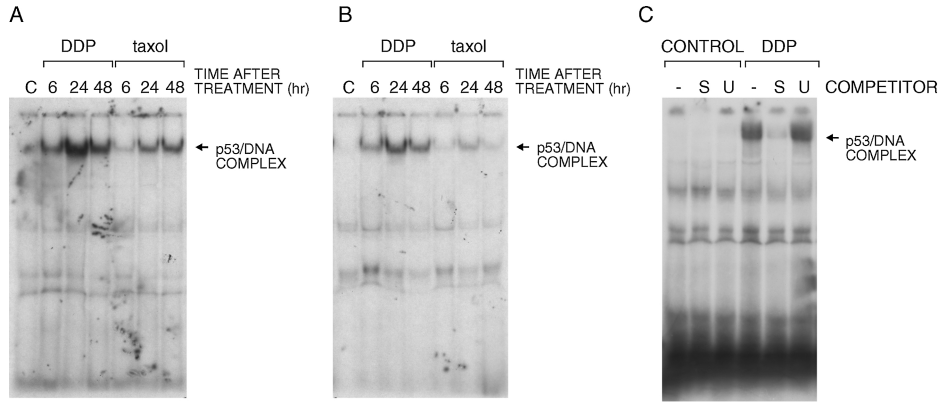
Figure 3. *Gel shift assay with extracts obtained from HCT-116 cells treated with DDP or taxol and performed with two different oligonucleotides (CON, panel A and p21, panel B), both containing a p53 binding site. Panel C reports the analysis with the CON oligonucleotide performed in the presence of a 50-fold molar excess of unlabeled specific (S) or unspecific (U) oligonucleotide.*

MgCl2, 0.1% NP40, 5% sucrose, 1 μg poly(dIdC), 2 μl of pAb421 hybridoma supernatant, and 1 ng of ^{32}P−end-labeled oligonucleotide CON (containing the p53 binding site 5′-GGACATGCCCGGGCATGTCG-3′) or p21 (containing the p53 binding site 5′-CAACATGTTGGGACATGTTC-3′). DNA−protein complexes were separated by electrophoresis through a 5% native polyacrylamide gel, dried, and visualized.

DNA-PK and ATR Activities

Cell extracts for DNA-PK activity were obtained according to procedures described previously [31]. DNA-PK activity was assayed using a kit according to the manufacturer (SigmaTECT DNA-PK Assay System, Promega, Madison, WI) with the modifications described by Woo et al. [23]. Assays were performed in triplicate and the mean values±SD are shown.

ATR activity was determined after immunoprecipitation with anti-ATR antibody as recently described [32] using recombinant PHAS-I (Stratagene, La Jolla, CA) in the presence of ^{32}P-ATP, resolved in SDS-PAGE, and auto-radiographed.

Results

The human colocarcinoma cell line HCT-116 and the human ovarian carcinoma cell line A2780, both expressing wt p53, were treated for 24 hours with concentrations of taxol and DDP close to their respective IC50. Total cell extracts were then taken after 6, 24, and 48 hours of incubation in drug-free medium. A typical Western blot analysis, performed in HCT-116 cells, is reported in Figure 1*A*. Both drugs induced an increase in the levels of p53. A marked phosphorylation at Ser15 of p53 was observed after DDP treatment but not after
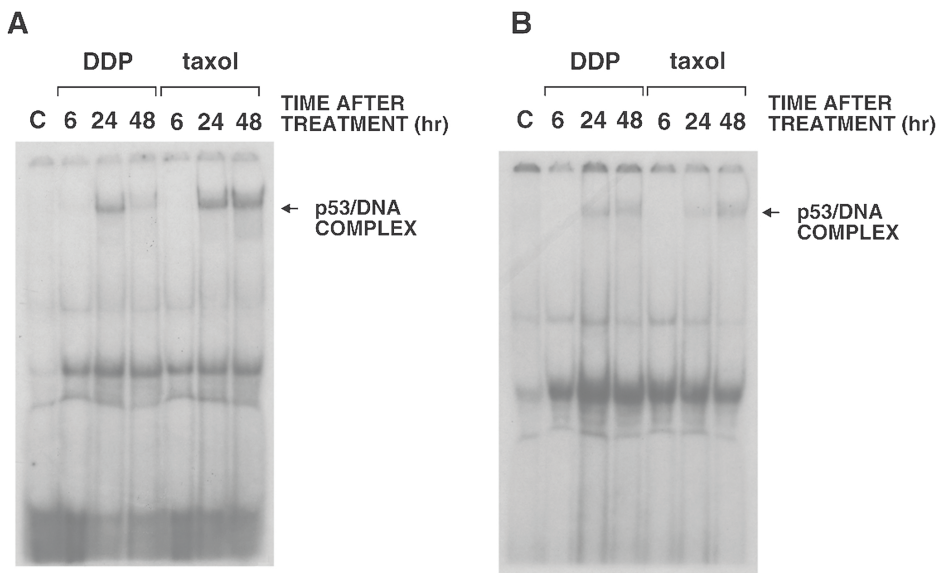

Figure 4. *Gel shift assay with extracts obtained from human ovarian cancer A2780 cells treated with DDP or taxol. Experiments were performed with two different oligonucleotides (CON, panel A, and p21, panel B).*

taxol treatment. Both drugs were able to induce phosphorylation at Ser20.

Tests were then performed to see whether the different phosphorylations of p53 induced by DDP and taxol could result in a differential activation of downstream genes. Levels of p21 and mdm-2 proteins following treatment with the two drugs were tested by Western blotting. Both DDP and taxol induced an increase in the levels of p21 and mdm-2 in HCT-116 cells, with DDP giving a higher p21 signal at 24 and 48 hours compared to taxol.

These findings were then extended to another cell system, the human ovarian cancer cell line A2780. Again, Ser15 of p53 was clearly phosphorylated after treatment with DDP but not after taxol treatment, under conditions in which a comparable level of phosphorylation at Ser20 and increased p53 levels were found after both drug treatments (Figure 2). A2780 cells were treated with the proteosome inhibitor LLnL, which stabilizes p53 protein levels by inhibiting degradation. In these conditions, no bands could be observed after incubation with both Ser15 and Ser20 phosphoantibodies, despite p53 amounts comparable to those induced after DDP or taxol treatment. As in the HCT-116 cell line, increased levels of p21 were observed after treatment with both drugs, and again DDP treatment induced a greater accumulation; mdm-2 protein could not be detected in this cell line by Western blot analysis, even after immunoprecipitation followed by Western blot analysis (data not shown).

Using the same treatment conditions, we further analyzed the ability of p53 to bind DNA in gel shift experiments using two different oligonucleotides containing the p53 binding site as probes. With both the CON and p21 oligonucleotides, a retarded band was observed from extracts of HCT-116 cells treated with DDP or taxol (Figure 3A and B). This band was observable after the addition of the monoclonal antibody against p53 pAb421 and designated as p53/DNA complex. This complex was competed by an excess of unlabelled CON or p21 oligonucleotide but not with an excess of an unrelated oligonucleotide (Figure 3C). The binding of p53 to DNA was observed in extracts from both DDP- and taxol-treated cells, although a stronger band was observed with both the CON and p21 oligonucleotides when using extracts from DDP-treated cells (Figure 3A and B). When testing A2780 extracts from control and DDP- or taxol-treated cells, a retarded band was observed, although the overall complex seemed less than observed when using HCT-116−treated cell extracts (Figure 4A and B).

To gain insights into the pathways leading to treatment-induced phosphorylation at Ser15 and Ser20 of p53, we investigated the possible role of the kinases reportedly involved in these phosphorylation events. Possible candidates are the phosphoinositide 3-kinase−related kinases (PIKs) (DNA-PK, ATM, ATR) hCHK1 and hCHK2, all proteins involved in cell cycle checkpoints and cellular response to DNA damage [33−35]. We treated HCT-116 cell lines with wortmannin, a well-known inhibitor of the PIKs, as described in the Materials and Methods section. Despite the almost complete inhibition of DNA-PK activity in cellular

extracts (Figure 5, *panel A*), phosphorylation at Ser15, although quantitatively slightly lower, could be observed after the concomitant treatment of DDP and wortmannin (Figure 5, *panel B*). Under the same treatment conditions, Ser20 phosphorylation after DDP and taxol treatment was reduced but still present.

We then asked whether ATM-defective cells were able to phosphorylate p53 at Ser15 in response to DDP treatment. Normal B lymphoblasts (IARC1663) and ataxia telangiec-
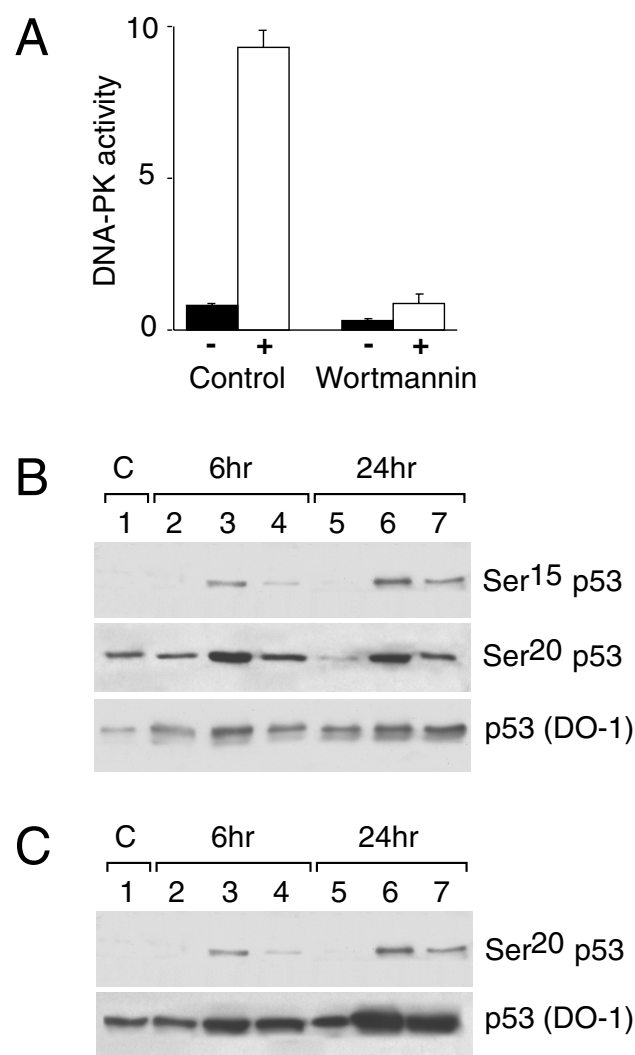

Figure 5. *HCT-116 cells were pretreated with wortmannin 35.5 μM for two hours before treatment with DDP or taxol. At the end of DDP or taxol treatment cells were washed in PBS and medium containing wortmannin (35.5 μM) was added. Panel A: Extracts for DNA-PK activity were obtained 8 hours after treatment with and without wortmannin and processed as described in Materials and Methods. Values are the means ± S.E. of pmolATP incorporated per minute per microgram protein in the biotinylated substrate in the absence (black columns) or in the presence of double-strand DNA (white columns). Panel B: Total cellular extracts were taken at 6 and 24 hours after treatment with wortmannin (lanes 2 and 5), with DDP (lanes 3 and 6), and with wortmannin and DDP (lanes 4 and 7). Blots were hybridized with antibodies recognizing p53 phosphorylated at Ser15, Ser20 and p53 (DO-1). Panel C: Total cellular extracts were taken at 6 and 24 hours after treatment with wortmannin (lanes 2 and 5), with taxol (lanes 3 and 6), and with wortmannin and taxol (lanes 4 and 7). Blots were hybridized with antibodies recognizing p53 phosphorylated at Ser20 and p53 (DO-1).*
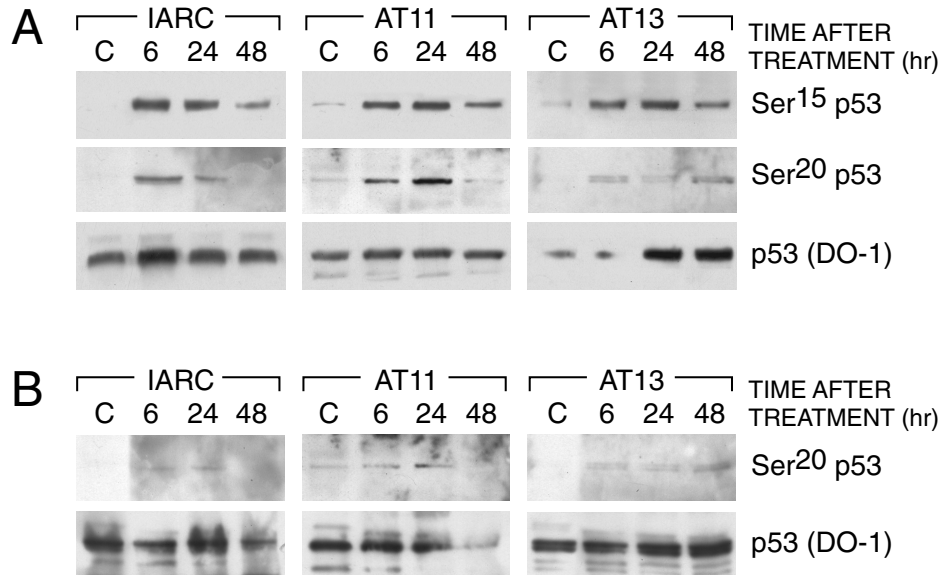
Figure 6. *Western blot analysis in IARC1663 and AT cell lines (AT11 and AT13) treated with DDP (Panel A) or taxol (Panel B). Extracts were taken at different times after treatment. Blots were hybridized with antibodies recognizing p53 phosphorylated at Ser15, Ser20 and p53 (DO-1).*
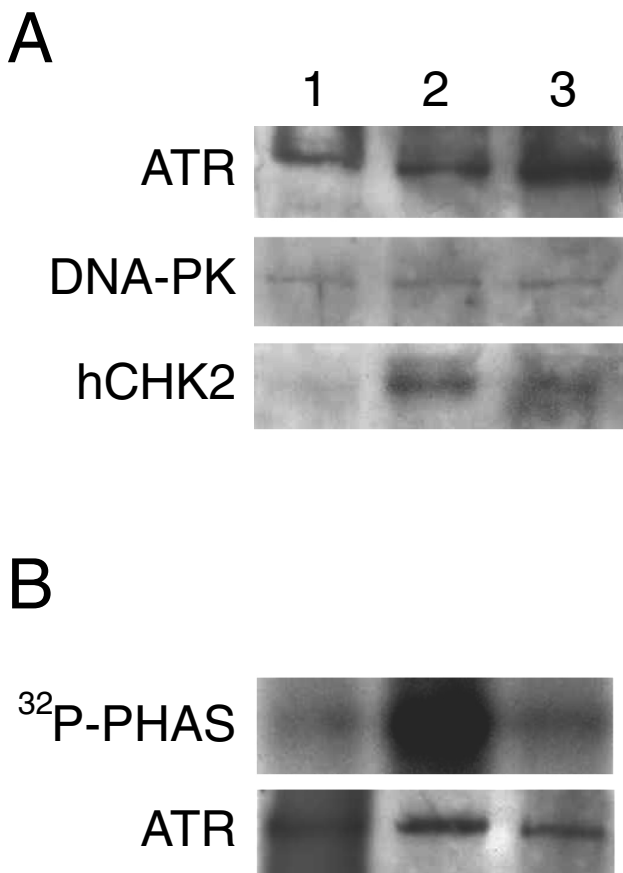

tasia (AT) lymphoblasts (AT11 and AT13 cell lines) were treated with 10 μM of DDP for two hours, a dose close to their IC50s. In response to DDP, p53 was phosphorylated at Ser15 both in normal and AT cell lines; this event was correlated with the induction of p53 (Figure 6). Similarly, phosphorylation at Ser20 was observable, either in wt IARC1663 cells or in AT-defective cells after both DDP and taxol treatment.

We finally investigated the effect of DDP and taxol on the levels and/or the activity of the kinases involved in Ser15 and Ser20 phosphorylation of p53. Figure 7*A* reports the Western blot analysis of HCT-116 cells treated with taxol or DDP. Whereas the levels of ATR and DNA-PK did not change after treatment with either drug, only DDP was able to induce ATR kinase activity (Figure 7*B*). Both DDP and taxol induced activation of hCHK2, as shown by a change in its intensity and electrophoretic migration (Figure 7*A*).

Figure 7. *Panel A: Western blotting analysis in HCT-116 cells untreated (lane 1) and treated with DDP (lane 2) or taxol (lane 3). Extracts were obtained 24 hours after treatment. Panel B: Activation of ATR kinase activity by DDP (lane 2) but not by taxol (lane 3) 24 hours after treatment in HCT-116 cells (lane 1 reports untreated cells). ATR was immunoprecipitated and kinase activity was assessed using PHAS substrate as described in the Materials and Methods section. The amounts of ATR present in each reaction were determined by Western blot and are shown at the bottom of the figure.*

Discussion

p53 is a critical mediator of cellular response to different stress conditions [1,2]. Even through p53's role in mediating cellular sensitivity and resistance to anticancer agents has been extensively studied, the results obtained so far in different cell systems are still quite controversial. This is particularly true for two widely used and effective anticancer agents, DDP and taxol. The cellular targets of DDP and taxol are very different, DNA for the former and tubulin for the latter [9,10]. Besides their different mechanisms of action, both drugs have been shown not only to activate p53 in different systems to a comparable level, but also to activate p53 downstream genes such as *p21* and *bax*.

The amount of data regarding the signal transduction pathways leading to activation of p53 after cellular damage is increasing. It is now clear that posttranslational modification

of p53, either phosphorylation or acetylation at its amino and carboxy-terminal domains are important for the activation of p53 as a transcriptional factor [20,36–38]. These post-translational modifications are thought to play a crucial role in determining the specificity of the p53 downstream genes activated and could represent an important determinant of the cellular decision between the p53-dependent cell cycle arrest or apoptosis. Interestingly enough, it seems that different types of damage utilize different sensors to achieve activation of p53. For example, the signalling mechanisms to p53 after IR damage are ATM dependent, but not after UV damage [24,25].

In the present study we focused on the Ser15 and Ser20 phosphorylation of p53 after treatment with two different anticancer agents DDP and taxol. Ser15 has been the first p53 site shown to be phosphorylated after DNA damage and was reported to be important for inhibition of p53 degradation by mdm-2 [19,20]. Ser20 phosphorylation has been reported to directly affect the binding between p53 and mdm-2 [39].

As already described for IR and UV damage, it was also found that treatment with DDP is able to activate a kinase that phosphorylates Ser15, whereas treatment with taxol is not. The reason why taxol failed to induce Ser15 phosphorylation in two different cellular systems is most likely due to its mechanism of action. This mechanism, not involving direct DNA damage (as in the case of DDP) is therefore not able to activate the putative kinases (particularly ATR) involved in the phosphorylation at this site. Ser15 of p53 has, in fact, been shown to be phosphorylated *in vitro* by the DNA-PK, ATM, and ATR kinases, all belonging to the PI3K family [33,40,41]. ATM is unlikely to be the kinase activated by DDP as judged by the Ser15 phosphorylation obtained in AT cell lines. Similarly, for DNA-PK there was evidence that, in spite of an almost complete inhibition of its activity by wortmannin, phosphorylation of Ser15 could be observed.

The results show that ATR kinase activity is clearly enhanced by DDP but not by taxol and this supports that the Ser15 phosphorylation is induced by DDP but not by taxol. These data are in concordance with recent observations that ATR effectively phosphorylates Ser15 of p53 *in vivo* after DNA damage [33,42].

Both DDP and taxol are able to induce phosphorylation of Ser20, which likely is responsible for the release of mdm-2 from p53 and the subsequent p53 stabilization observed after treatment with both drugs. The kinase responsible for the phosphorylation of Ser20 *in vivo* has been recently characterized as the human homologue of the yeast cds1/chk2 (hCHK2).

When checked whether the different p53 phosphorylations induced by DDP and taxol could result in different activations of p53 downstream genes, it was found that both drugs are able to induce increased levels of p21 and that DDP treatment was a better p21 inducer than taxol. The results show that both drugs are able to activate hCHK2, which phosphorylates p53 at Ser20, an event necessary for p53 stabilization [28–30], but that only DDP is able to activate ATR and to induce Ser15 phosphorylation. This is

likely to be responsible for the stronger p21 downstream activation induced by DDP, because Ser15 phosphorylation has been reported to stabilize p53/DNA binding [43,44].

Although it is plausible that, *in vivo*, different kinases could participate in phosphorylation of p53 (particularly at the sites investigated here) and that it could therefore be difficult to identify a single kinase activated by a stress signal, knowledge of the different signal transduction pathways and mechanisms of p53 activation used by anticancer agents with different mechanisms of action might help in designing not only critical and specific modulators of p53 activity, but also in identifying new therapeutic targets.

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