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Activation of p53 by the cytoprotective aminothiol WR1065: DNA-damage-independent pathway and redox-dependent modulation of p53 DNA-binding activity

Olivier Pluquet[a], Sophie North[a,1], Marie-Jeanne Richard[b], Pierre Hainaut[a,*]

[a]*Unit of Molecular Carcinogenesis, International Agency for Research on Cancer, 69372 Lyon Cedex 08, France*
[b]*UF Cancérologie Biologique et Biothérapie, Centre Hospitalier Universitaire Albert Michallon, 38043 Grenoble Cedex 03, France*

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Abstract

WR1065 is an aminothiol with selective cytoprotective effects in normal compared to cancer cells, which is used to protect tissues against the damaging effect of radiation and chemotherapeutic drugs. WR1065 has been shown to induce wild-type p53 accumulation and activation in cultured cells, suggesting a role of p53 in cytoprotection. However, the molecular mechanisms by which WR1065 activates p53 remain unclear. Here, we demonstrated that p53 accumulation by WR1065 in MCF-7 cells did not result from the formation of DNA-damage as measured by DNA fragmentation and Comet assay, nor from oxidative stress as detected by measurement of glutathione levels, lipid peroxidation and reactive oxygen species production. p53 activation by WR1065 was not prevented by inhibition of PI-3 kinases, and was still detectable in MCF-7 cells stably transfected with the oncoprotein E6, which repressed p53 induction by DNA damage. These data provided evidence that WR1065 induces p53 by a pathway different than the one elicited by DNA-damage. Direct reduction by WR1065 of key cysteines in p53 may play an important role in this alternative pathway, as shown by the fact that WR1065 activated the redox-dependent, DNA-binding activity of p53 *in vitro*.

Keywords: WR1065; p53; Cytoprotection; Polyamines; Oxidation–reduction; DNA damage pathway

1. Introduction

WR1065 is an aminothiol produced by dephosphorylation of (*S*-2[3-aminopropylamino]-ethylphosphothioic acid, WR2721, Ethyol®) by membrane-bound alkaline phosphatase [1]. WR1065 is a wide spectrum tissue protector currently used as a protective agent in cancer patients receiving radio or chemotherapy [2–4]. *In vivo*, WR1065 protects normal cells against toxicities induced by radiation, alkylating or platinum agents [5–7], without impairing the efficacy of radio- and chemotherapy [8]. The molecular basis for the selectivity of WR1065 for healthy tissues, as well as for its protective action, are still poorly understood. Several effects of WR1065 have been described that are relevant for cytoprotection. They include the ability to scavenge free radicals [9], to participate in hydrogen donation processes to repair oxidized DNA, or to exert anticlastogenic and antimutagenic effects in cultured cells [10,11]. However, others mechanisms including stimulation of hematopoietic progenitors [12,13], or a protection through an hypoxymimetic effect [14], suggest that direct radical scavenging may not be the only mechanism of action of this drug. *In vitro*, WR1065 induces a reduction in cell proliferation [15–17], down regulates several genes involved in cell proliferation including thymidine kinase [18] and c-myc [19] and increases the expression of genes involved in redox regulation such as NF-κB and MnSOD [18–20].

We have recently shown that WR1065 induces the accumulation and activation of the tumor suppressor protein p53 [21]. p53 is a transcription factor which controls a complex pattern of responses to genotoxic, non genotoxic

* Corresponding author. Tel.: +33-472-738-532; fax: +33-472-738-322.
E-mail address: hainaut@iarc.fr (P. Hainaut).
[1] Present address: Laboratoire des Facteurs de Croissance, INSERM EPI 0113, Université de Bordeaux 1, 33405 Talence, France.

Abbreviations: AG, aminoguanidine; GSH, reduced glutathione; WR1065, 2-(3-aminopropylamino)ethanethiol; DTT, dithiothreitol; EMSA, electrophoretic gel mobility-shift assay; PI-3-K, phosphatidylinositol-3 kinase; AT, ataxia telangectasia; HPV, human papilloma virus.

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and oncogenic stresses induced by multiple stimuli [22,23]. In response to these stresses, p53 undergoes multiple post-translational modifications, which stabilize the protein and turn it from a latent to an active form with a high affinity for specific DNA sequences. It has been also reported that binding of p53 to DNA is dependent on the reduced state of critical cysteine residues located at the DNA-binding surface [24]. The transactivation of p53 target genes leads to the control of cell proliferation, survival and genetic stability [25–27]. It has been shown that WR1065 protects normal fibroblasts from cytotoxicity of paclitaxel in a p53-dependent manner [28]. Thus p53 induction may play a role in the cytoprotective effects of WR1065. Alternatively, this phenomenon may be the hallmark of the capacity of WR1065 to generate a potentially damaging genotoxic stress.

Here, we first demonstrate that induction of p53 by WR1065 is not the consequence of the formation of DNA-strand breaks, nor of oxidative stress. Second, we show a direct thiol-reducing effect of WR1065 in the activation of p53 *in vitro*. These results indicate that WR1065 induces p53 by a stress signaling pathway independent of DNA-damage and that direct reduction by WR1065 may increase p53 DNA-binding activity.

2. Materials and methods

2.1. Cell culture and treatments

The breast carcinoma cell lines MCF-7 (expressing wild-type functional p53) and MCF-7/E6 (expressing wild-type p53, partially inactivated by the human papilloma virus protein E6), were kindly provided by A.J. Fornace (Bethesda) and cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL). MCF-7/E6 cells were maintained by selection with 0.4 mg/mL G418 (Life Technologies). The human lymphoblastoid cell lines AT-11 (ATM-deficient) and 2139 (ATM-competent) were kindly provided by J. Hall (IARC) and were cultured in RPMI (Gibco-BRL). Cells were maintained at 37° with 10% fetal calf serum (PAA), 2 mM L-glutamine and antibiotics, under 10% CO_2 (MCF-7) or 5% CO_2 (lymphoblastoid cell lines).

Adherent cells were treated with drugs at 50–65% confluency while cells growing in suspension were exposed to agents at a cell density of 1×10^6 cells/mL. WR2721 and WR1065 were provided by USB Pharma, dissolved in phosphate-buffered saline (PBS), and WR1065 solutions were flushed with argon to prevent oxidation. Aminoguanidine (Sigma) was dissolved in culture medium and added to cells 10 min before WR1065. Wortmannin (Sigma) was dissolved in DMSO and used at 200 nM. Cells were pretreated with wortmannin for 30 min before exposure to aminoguanidine and WR1065. Hydrogen peroxide and spermidine (Sigma) were dissolved in deionized water. JC-01 was from Molecular Probes and dissolved in DMSO (1 mg/mL as stock solution).

2.2. Protein extraction, Western blot and antibodies

Cells were washed twice with ice-cold PBS and collected by scraping (adherent cells) or centrifugation (lymphoblastoid cells). Protein extracts were prepared as described [29]. Briefly, cells were lysed for 15 min on ice in 100 μL (per million cells) buffer-A (20 mM HEPES (pH 7.6), 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 0.1% NP40), containing protease inhibitors (0.5 mM PMSF, 0.5 μg/mL leupeptine, 2 μg/mL aprotinin, 0.7 μg/mL pepstatin A, 1 mM sodium fluoride and 50 μM sodium orthovanadate). After centrifugation at 300 *g* for 4 min, the supernatant was designated "cytoplamic fraction" and stored at −80°. The pellets were further extracted for 30 min on ice in 50 μL (per million cells) buffer-B (same as buffer-A, with 0.5 M instead of 10 mM NaCl) and centrifuged for 15 min at 15,000 *g* and 4°. The supernatant was designated "nuclear fraction" and stored at −80°. Total protein extracts were performed in buffer-B, supplemented as described above.

Equal amounts of proteins (quantified by Bradford assay) were mixed with Laemmli sample buffer, resolved on 10% SDS–polyacrylamide gel, and transferred to PVDF membranes (Roche). Proteins were revealed by using an enhanced chemoluminescence detection system in accordance with the manufacturer's instructions (ECL, Amersham). For p53 detection, antibody DO-7 (1:1000, DAKO) was used. Anti-actin monoclonal antibody (C-2, 250 ng/mL) was from Santa Cruz (Santa Cruz, CA). Peroxidase-conjugated goat anti-mouse IgG (250 ng/mL, Pierce) was used as secondary antibody.

2.3. Electrophoretic gel mobility-shift assay (EMSA)

The double-stranded p53 consensus binding sequence $p53^{con}$ (5′-GGACATGCCCGGGCATGTCC-3′) was end-labeled with ~3000 Ci/mmol [γ-^{32}P] ATP (Amersham) as described [29]. Baculovirus-expressed human p53 was produced in S*f*-9 and purified in buffers depleted of transition metals and DTT as described previously [30]. Human recombinant p53 protein (25 ng) was renatured with $ZnCl_2$ (200 μM) and either DTT, WR2721 or WR1065 (all at 4 mM) in EMSA buffer containing NaCl (140 mM) for 20 min at 37°. Binding reactions contained ^{32}P-labeled double-stranded oligonucleotide (0.5 ng), sonicated herring-sperm DNA (2 μg; Promega), BSA (5 μg), and either DTT, WR1065, WR2721 or spermidine at 4 mM. Reactions were adjusted to a final volume of 30 μL with buffer-A. All reactions were carried out in the presence of the monoclonal antibody PAb 421 (100 ng per reaction). This antibody stabilizes p53–DNA complexes and is required to detect stable binding of p53 to short oligonucleotides in cellular extracts. No band was detected in the absence of

PAb 421. The same binding protocol was used with nuclear protein extracts (10 μg), except that all binding reactions contained DTT at 4 mM [29]. Binding reactions were incubated for 30 min at 20°. A 15 μL aliquot of each reaction was loaded onto a 4% non-denaturing polyacrylamide gel and run in TBE buffer at 120 V for 2–3 hr. Gels were fixed, dried and exposed to KODAK X-ray films at −80° for 12–48 hr. The specificity of the binding was controlled by competition experiments using cold oligonucleotides and using mutant DNA consensus sequence as described previously [29].

2.4. Detection of DNA damage

MCF-7 cells were grown in 24-well plates at 37°. After incubation with aminoguanidine (4 mM) and WR1065 (1 mM) with or without H_2O_2 (1 mM), DNA fragmentation was evaluated using the DNA fragmentation ELISA kit (Boehringer) according to the manufacturer's instructions, with the following modifications: (i) labeling of cells with BrdU was performed during 1 hr at the final concentration of 10 μM before treatment with drugs; (ii) cells were lysed by adding 400 μL per well of incubation buffer. Results were expressed as relative absorbance at 405 nm.

For Comet assays, MCF-7 cells were treated in Petri dishes with aminoguanidine (4 mM) for 10 min prior to addition of WR1065 (1 mM), or with H_2O_2 (150 μM) for 2 hr. Cells were trypsinized and resuspended in PBS. The procedure described in [31] was modified as follows: about 2×10^4 cells were mixed in 1.2% low melting point agarose (FMC Bioproduct) and poured on agarose-coated slides. After lysis in the dark in 100 mM Tris (pH 10.0), 1% Triton X-100, 10% DMSO, 2.5 mM NaCl, 100 mM Na_2EDTA, 10% sodium laurylsarcosinate, cells were examined by epifluorescence microscopy (Zeiss Axioskop 20, Carl Zeiss), equipped with a mercury lamp HBO (50 W, 516–560 nm, Zeiss) and filters 5 and 15 (Zeiss) at 20× magnification. Each experimental condition was processed in triplicate. Fifty randomly selected comets on each triplicated slides were scored with a pulmix TM 765 camera and analyzed with the Komet 3.0 image analysis system (Kinetic Imaging).

2.5. Measurement of intracellular H_2O_2 and redox parameters

Fluorometry was used to measure intracellular H_2O_2 production with 2′,7′-dichlorofluoresceine diacetate (DCF-DA) (Molecular Probes). MCF-7 cells were grown in 24-well plates (Beckton Dickinson) and exposed to drugs at the indicated times in presence of 5 μM DCF-DA. H_2O_2 (500 μM) was used as positive control. Cells were washed in PBS and the intensity of fluorescence was measured by fluorometry (Fluoroskan, Lab Systems) using 485 nm for excitation and 525 nm for emission. Levels of oxidized and reduced glutathione were determined as described by Akerboom and Sies [32], with modifications [29]. Products of lipid peroxidation were evaluated after acid hydrolysis at 95° to form malondialdehyde (MDA), which reacts with thiobarbituric acid to yield a MDA–TBA_2 adduct measured by fluorimetry.

2.6. Measurement of mitochondrial depolarization ($\Delta\psi_m$)

$\Delta\psi_m$ was analyzed by JC-01 fluorescence in intact MCF-7 cells cultured in Labtech slides (Nalge Nunc International). Cells treated with WR1065 (1 mM + AG) for 24 hr or H_2O_2 (1 mM) for 12 hr, were incubated for 20 min in the dark, at 37° with JC-01 (20 μg/mL in medium as final concentration), and then washed with PBS. The mitochondria were visualized by epifluorescence microscopy using a 520 nm longpass optical filter. JC-01 is a lipophilic cation that selectively crosses the negatively charged membrane of mitochondria, forms J-aggregates in the matrix of mitochondria or is released in the monomeric form from depolarized mitochondria. Mitochondrial polarization is indicated by red fluorescence due to J-aggregate formation by the concentrated dye. Mitochondrial depolarization ($\Delta\psi_m$) is indicated by green fluorescence of the JC-01 monomers.

2.7. Statistical evaluation

For autoradiograms, densitometric quantification was performed using a Bio-Rad imaging densitometer GS-670 and Molecular Analyst software (Bio-Rad). The significance of observed differences was evaluated using the two-tailed t student's test. Probabilities of $P < 0.05$ were regarded as statistically significant.

3. Results

3.1. Effect of WR1065 on damage formation in MCF-7 cells

We have previously shown that WR1065 induced the accumulation and activation of the tumor suppressor protein p53 [21]. Since genotoxic stress represents the primary mechanism of p53 induction, we examined whether exposure of WR1065 to MCF-7 cells induced the formation of DNA-strand breaks, using a DNA fragmentation assay to detect the release of nucleosomal fragments. All experiments were performed in presence of aminoguanidine (AG) at a concentrations of 4 mM as determined previously [21]. Indeed, it has been reported by Meier and Issels [33] that in the culture medium, WR1065 may undergo degradation into cytotoxic metabolites (H_2O_2, acrolein) by copper-dependent amine oxidases.

Time-course experiments with MCF-7 cells indicated that WR1065 (1 mM + AG) did not generate detectable DNA fragmentation after up to 8 hr of treatment (Fig. 1A).

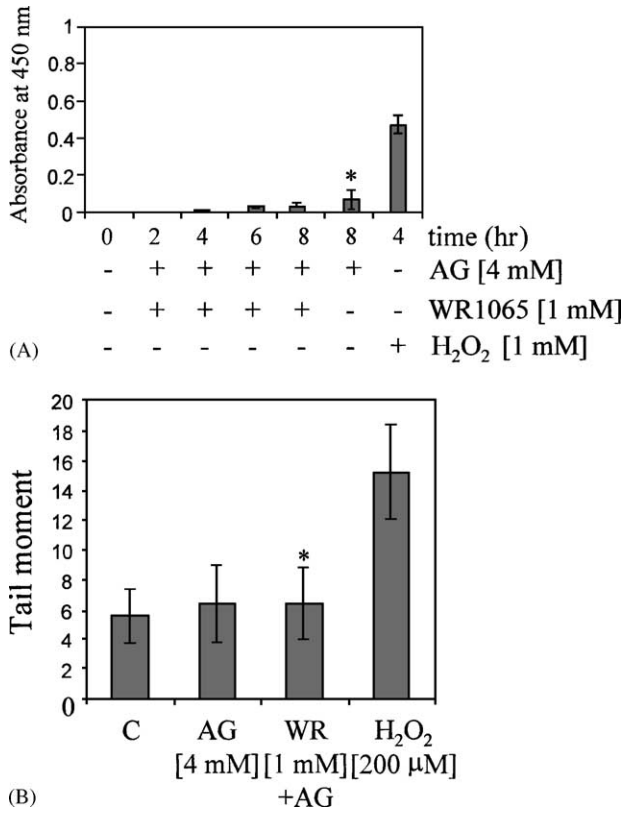

Fig. 1. (A) Absence of DNA-strand break damage after treatment with WR1065. MCF-7 cells were pre-treated for 10 min with AG (4 mM), then exposed to WR1065 (1 mM) for up to 8 hr. As a positive control, H_2O_2 (1 mM) was added for 4 hr. DNA fragmentation was evaluated by ELISA detection of BrdU-labeled DNA fragments in cell lysates (∗: $P < 0.05$). (B) Evaluation of strand break damage using Comet assay. MCF-7 cells treated with either AG, WR1065 (1 mM + AG) or H_2O_2 (200 μM) were tested. Yield of damage is represented by the mean tail moment of 50 cells/slides (∗: P = N.S., N = 3).

These conditions were shown to induce a high and sustained accumulation of p53 [21]. Similar results were obtained with another cell line, TE-1 (data not shown). To further detect the formation of subtle, low levels of initial DNA-damage, we performed Comet assays in MCF-7 cells exposed for 2 hr to WR1065 (1 mM + AG) (Fig. 1B). Results show that WR1065 did not induce any significant increase in the mean tail moment of the comets. Together, these data indicate that in these conditions, WR1065 did not generate detectable DNA-strand break damage.

Although WR1065 is known as a radical scavenger, it may interfere with intracellular redox reactions to induce the formation of oxidative stress, which may in turn trigger p53 activation. However, using a fluorescent probe to detect intracellular accumulation of peroxide, we did not find any elevation of oxyradicals in cells treated with WR1065 (1 mM + AG) (Fig. 2A). As one of the major sources of free radicals in cells is mitochondrial leakage, we next investigated the effects of WR1065 on mitochondrial membrane potential. MCF-7 cells were treated with H_2O_2 for 12 hr, WR1065 (1 mM + AG) for 24 hr and incubated with JC-01, which the ratio green to red fluorescence depends on mitochondrial membrane potential, did not change in WR1065-treated cells compared to untreated cells (Fig. 2B). In contrast, cells exposed to H_2O_2 (at a concentration inducing cell killing, 1 mM) exhibited a shift in fluorescence emission from red to green indicating mitochondrial membrane depolarization. Moreover, we did not detect any increase in the formation of lipid peroxidation products (Fig. 2C) and levels of GSH showed a time-dependent increase with a maximum at 12 hr (Fig. 2D). Interestingly, control experiments performed with WR1065 in the absence of AG did not show an increase in peroxide production or mitochondrial depolarization, suggesting that the degradation of WR1065 does not significantly contribute to the production of radical damage in our experimental conditions. Together, these observations indicate that WR1065 did not induce detectable intracellular oxidative stress in conditions where the p53 protein is activated.

3.2. *Effect of WR1065 on the pathway of p53 induction by DNA-damage*

The process of p53 activation after DNA damage involves phosphorylation of p53 by pathways controlled by large protein with PI-3 kinase domains, such as ATM and ATR. To explore the role of PI-3 kinase activities in the induction of p53 by WR1065, we used wortmannin, an inhibitor of most PI-3 kinases. Addition of wortmannin to cells exposed to WR1065 did not prevent accumulation of p53 and activation of DNA-binding capacity (Fig. 3A). In contrast, wortmannin prevented accumulation and activation of p53 in response to hydrogen peroxide. As a control for inhibition of PI-3 kinase activity, we verified that wortmannin prevented the phosphorylation of the PI-3 kinase substrate Akt after stimulation with H_2O_2 (data not shown). Overall, these results suggest that kinases with PI-3 kinase domains were unlikely candidates in the pathway of induction of p53 by WR1065.

To specifically rule out a possible role of ATM, we determined the level of p53 in AT-11 cells, a lymphoblastoid cell line established from an AT patient expressing a truncated ATM gene. While AT-11 showed a delayed and decreased response to hydrogen peroxide, this cell line exhibited a normal p53 response to WR1065 (Fig. 3B).

To further compare the process of p53 induction in response to WR1065 and to DNA-damage, we used MCF-7 cells stably transfected with E6 of HPV18. The E6 protein of oncogenic forms of HPVs binds to p53, induces its proteasome-dependent degradation through a pathway which does not require Mdm2, and thus prevents p53 induction in response to DNA-damaging agents [34]. Results show that E6 did not prevent induction of p53 by WR1065 (Fig. 3C). These results further support that WR1065 induced p53 through a stabilization pathway distinct from the one activated by DNA-damaging stimuli.

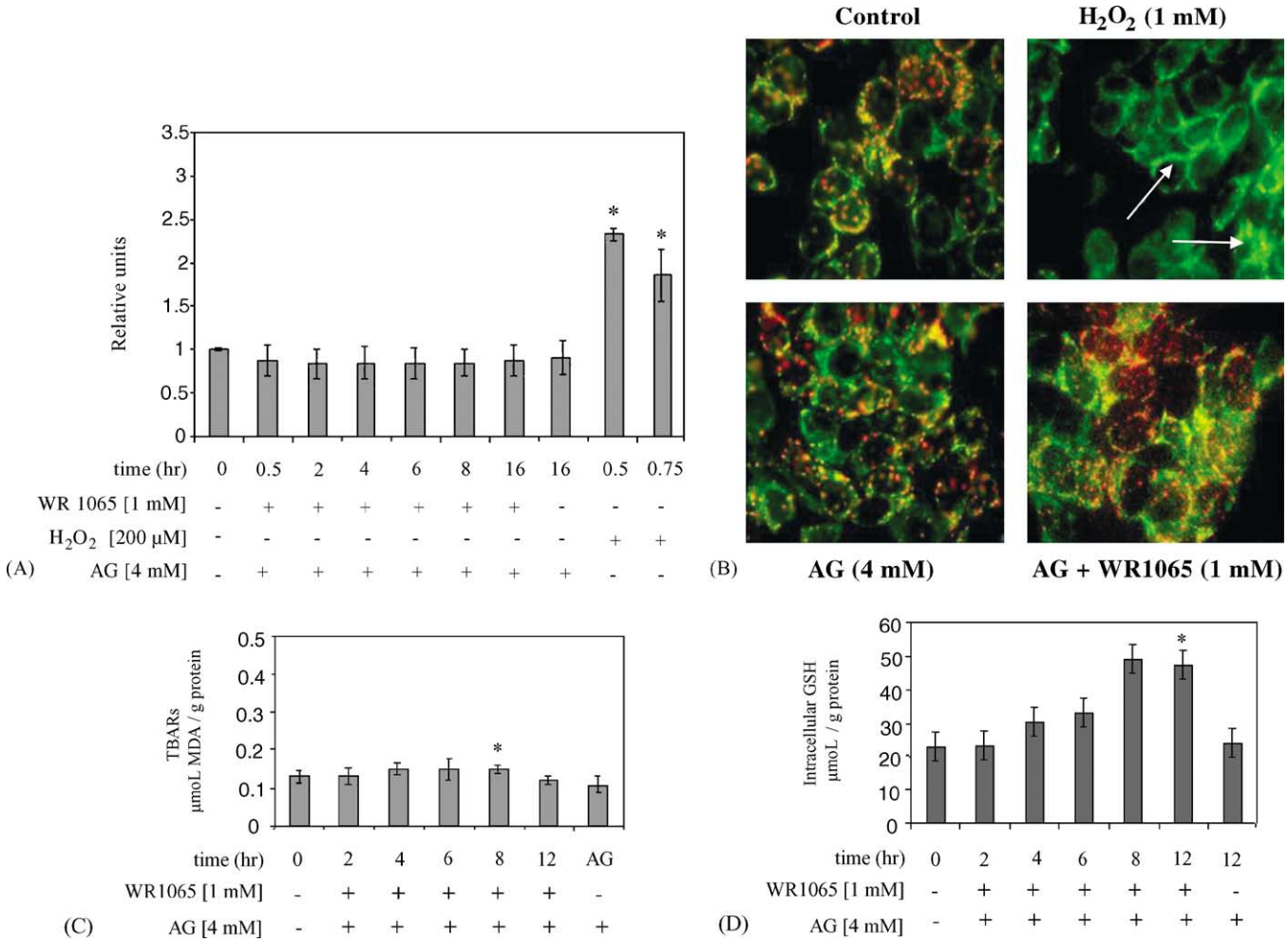

Fig. 2. (A) Absence of detectable intracellular oxidative stress after treatment with WR1065. Detection of intracellular reactive oxygen species using the fluorescent probe H_2DCFDA. MCF-7 cells were treated with WR1065 (1 mM + AG) or with H_2O_2 (200 µM) for different times as indicated. After treatment, fluorescence levels in attached cells were measured at 485 nm for excitation and 515 nm for emission. Results are expressed as relative values, a value of 1 corresponding to the level of fluorescence in untreated cells (∗: $P < 0.05$). (B) Effect of WR1065 in mitochondrial membrane potential ($\Delta\psi_m$). MCF-7 cells were pre-treated for 10 min with AG, then exposed to WR1065 (1 mM) for 24 hr or to H_2O_2 (1 mM) for 12 hr. Cells were then incubated with JC-01 and observed under epifluorescence microscopy (magnification 60×). A shift from green to red fluorescence (white arrows) indicates disruption of $\Delta\psi_m$. (C) Levels of lipid peroxidation in MCF-7 cells exposed to WR1065 (1 mM + AG) (∗: P = N.S.). Results are expressed in µmoles of malondialdehyde–thiobarbituric adducts (MDA–TBARs)/g of cellular protein extracts. (D) Intracellular levels of reduced glutathione in MCF-7 cells treated with WR1065 (1 mM + AG). Results are expressed in µmol GSH/g total cellular proteins (∗: $P < 0.05$). In each graphs, bar represents the average ± SD of at least three independent experiments.

3.3. Effect of WR1065 on p53 activation upon exposure of MCF-7 cells to H_2O_2

WR1065 has been shown to protect cells against H_2O_2 injury [35]. In Fig. 4A, we show that WR1065 significantly decreased by 50% the DNA fragmentation resulting from exposure of hydrogen peroxide. Since H_2O_2 is a strong inducer of p53 through DNA-damage, it should be expected that scavenging by WR1065 would strongly reduce the extend of p53 activation in response to H_2O_2. However, in Fig. 4B, we show that, despite the observed decrease in DNA fragmentation, p53 accumulation and activation is similar in cells treated with H_2O_2 alone or with H_2O_2 and WR1065. These results suggest that p53 induction by WR1065 occurs through a mechanism which is dominant over the one induced by H_2O_2.

3.4. Stimulation of p53 DNA-binding activity in vitro by WR1065

The p53 protein is intrinsically sensitive to oxidation–reduction, and binding to DNA *in vitro* requires the reduction of several cysteines located within the contact surface between the protein and specific DNA motifs [24]. It has been suggested that WR1065 can bind to p53 *in vitro* [28]. To determine whether WR1065 can affect p53 DNA-binding through a redox mechanism, we used an *in vitro* binding assay with a recombinant wild-type p53 [30]. In this assay, binding of p53 to specific DNA is dependent upon addition of zinc and of thiol reducing agents such as DTT [36,37]. We have previously shown that this recombinant p53 protein does not bind DNA unless renatured by incubation with $ZnCl_2$ (50–200 µM) and DTT (5 mM) [30]

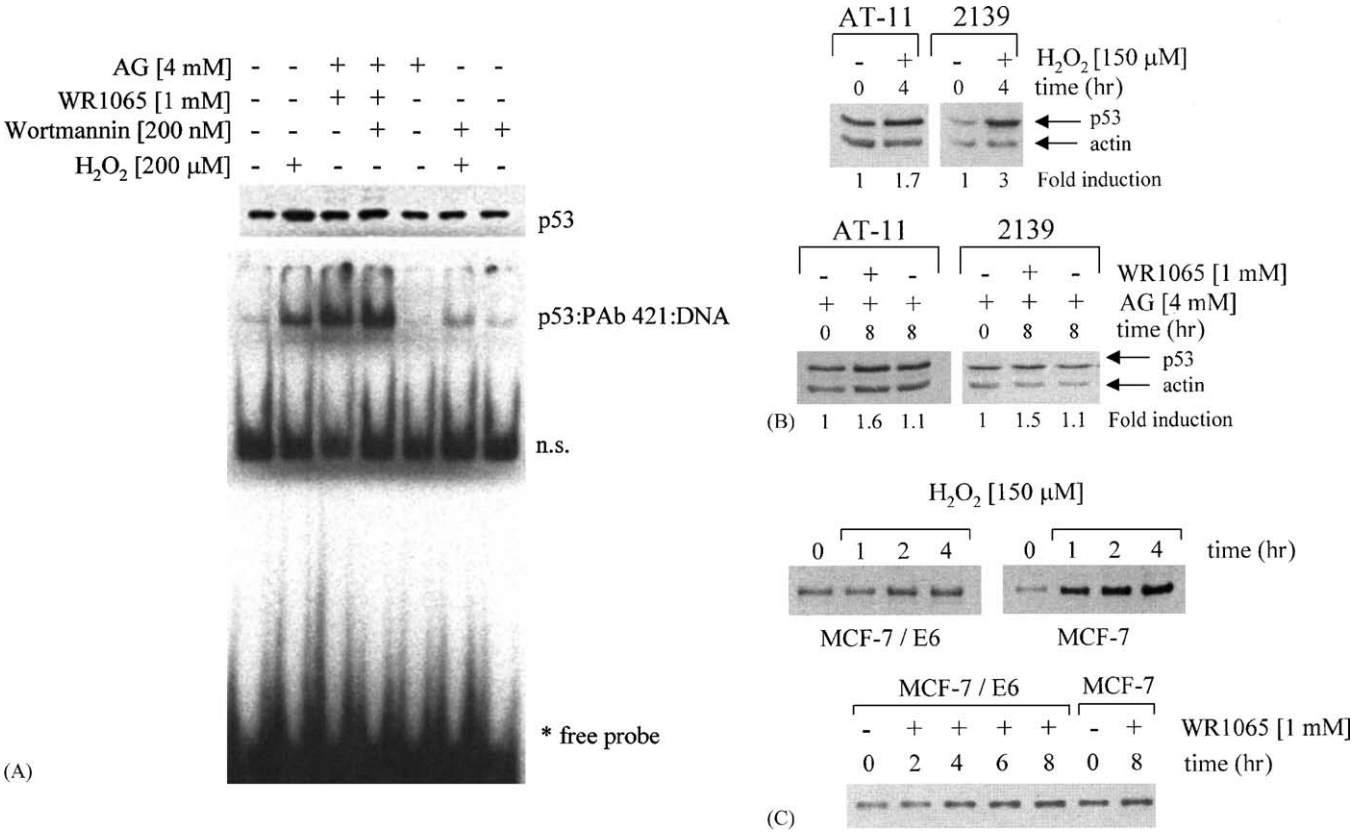

Fig. 3. WR1065 induces p53 by a pathway independent of DNA damage. (A) Effects of PI-3 kinase inhibition. MCF-7 cells were pre-treated with the PI-3 kinase inhibitor wortmannin (200 nM) for 30 min and then exposed to AG (4 mM) or WR1065 (1 mM + AG) for 2 hr. Nuclear protein extracts were used to determine p53 level by Western-blot using DO-7 antibody. p53 DNA-binding activity was determined in same nuclear extracts, the asterisk indicates the free probe, and n.s. (non-specific band) corresponds to an already described, shifted band which does not correspond to p53 [29]. (B) Role of ATM in response to WR1065. Total protein extracts were prepared at different times after treatment with WR1065 (1 mM + AG) or H_2O_2 (150 μM) from two lymphoblastoid cell lines. Western-blot was performed using DO-7 antibody and actin antibody as internal control. (C) MCF-7/E6 cells and MCF-7 cells were exposed to H_2O_2 for up to 4 hr, or to WR1065 (1 mM) and the level of p53 protein was detected in nuclear extracts by Western-blot using DO-7.

(Fig. 5A). We compared the capacity of DTT, WR1065, the phosphothiol pro-drug WR2721, and the polyamine spermidine to activate p53 DNA-binding *in vitro*. Fig. 5B shows that WR1065 stimulated DNA-binding to a higher degree than DTT. In contrast, WR2721 and spermidine did not activate DNA-binding. These results indicate that WR1065 efficiently reduced p53 and enhanced its redox-dependent binding to DNA, suggesting that this mechanism could in part explain the stabilization of p53 and its dominant effect over the one of H_2O_2.

4. Discussion

It has been proposed that the formation of DNA damage, either by direct or indirect mechanisms, was a common denominator of many stimuli inducing p53 [38]. Here, we present evidence that WR1065, the active metabolite of the chemoprotective aminothiol amifostine, activates p53 by a pathway which does not require the formation of DNA-damage. We also show that WR1065 can affect p53 protein activity by the modulation of direct sequence-specific binding to DNA.

Our results show that WR1065 can activate binding of p53 to specific DNA *in vitro* in a redox-dependent manner. WR1065 substitutes to thiol anti-oxidants such as DTT to renaturate recombinant p53 protein and to activate its binding to a consensus oligonucleotide. WR2721, the phosphorylated form of the drug, is unable to stimulate DNA-binding *in vitro*, indicating that the free thiol group is essential for this effect. Previous studies have shown that p53 is intrinsically redox-sensitive, and that the DNA-binding domain contains several cysteines (some of them involved in zinc binding) that need to be reduced for optimal DNA-binding [36,37,39]. Although we have no direct demonstration that WR1065 modifies the redox state of p53 *in vivo*, our observations are compatible with data showing that WR1065 can bind to recombinant p53 *in vitro* [28]. This interpretation is also in agreement with our recent observation that amifostine can stabilize p53 protein conformation, as shown by its capacity to convert a temperature-sensitive mutant p53 from "mutant" to "wild-type" phenotypes in both cultured cells and in a yeast functional assay [40,41]. Two other redox-dependent transcription factors, NF-κB and AP-1, have also been shown to be activated by WR1065 in *in vitro* assays, indicating

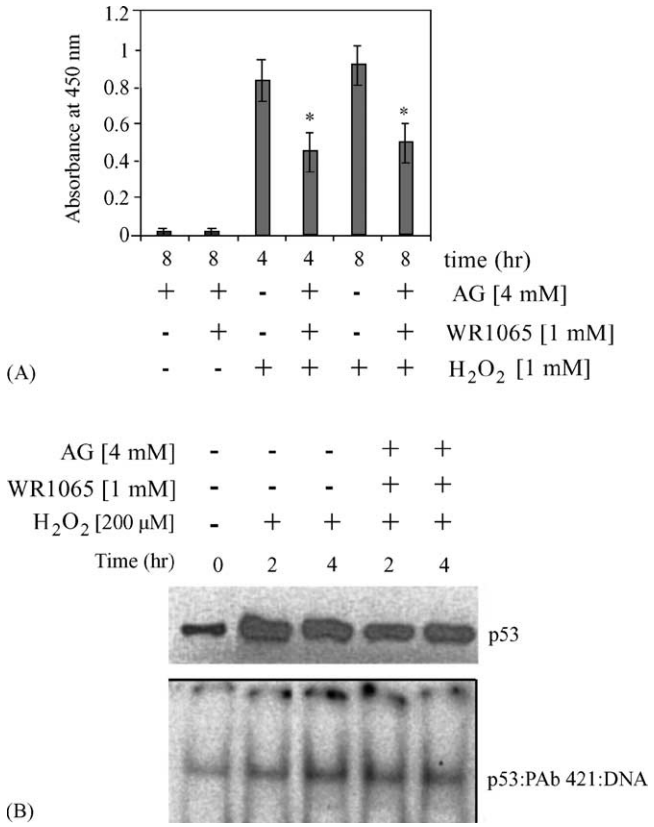

Fig. 4. p53 induction by WR1065 is dominant over the one induced by H_2O_2. (A) MCF-7 cells were pre-treated for 10 min with AG, then exposed to WR1065 (1 mM) for 4 or 8 hr, in absence or presence of H_2O_2 (1 mM). DNA fragmentation was evaluated by ELISA detection of BrdU-labeled DNA fragments in cell lysates (∗: $P < 0.05$). (B) MCF-7 cells were exposed to WR1065 (1 mM + AG) in presence or absence of H_2O_2 (200 μM). Equal amount of nuclear extracts were loaded to determine the level of p53 by Western blot. The DNA-binding activity of p53 was analyzed by EMSA (as in Fig. 3). Only the portion of the autoradiogram with the specific p53:PAb 421:DNA complexes is shown.

that the redox effect of WR1065 is not specific for the p53 protein [28].

The contribution of redox mechanisms to the induction of wild-type p53 in cells treated with WR1065 is not clear. Exposure of cultured cells to WR1065 induces p53 protein accumulation, suggesting that the drug not only modulates protein conformation but also interferes with the control of p53 protein stability. Thus, other mechanisms in addition to reduction of p53 need to be considered to explain the effects observed in cultured cells.

Our results support the notion that p53 induction in response to WR1065 occurs essentially through DNA-damage-independent mechanisms. First, WR1065 does not induce detectable DNA-strand breaks, as measured with two different techniques including Comet assays. Addition of WR1065 even significantly reduces the level of strand breaks induced by hydrogen peroxide. Second, WR1065 does not induce oxidative stress, as detected by measurements of reduced glutathione levels, mitochondrial depolarization, lipid peroxidation and reactive oxygen species production. Third, inhibition of PI-3 kinase activities fails to inhibit p53 induction by WR1065, although it significantly reduces the activation of p53 by hydrogen peroxide (Fig. 4) [42]. In agreement with the latter observation, a lymphoblastoid cell line established from an AT patient (expressing a truncated, dysfunctional ATM gene) shows a normal p53 response to WR1065, but a delayed and decreased response to hydrogen peroxide. Fourth, the notion that induction of p53 by WR1065 is not dependent upon DNA-damage is further supported by the observation that it is not prevented by HPV18 E6. In most cultured cells, the presence of E6 prevents wild-type p53 stabilization after DNA damage, but E6-independent p53 stabilization has been observed in response to non-DNA-damage stimuli such as hypoxia [34] and hypoxy-mimetic agents such as cobalt chloride (O. Pluquet, unpublished results). Thus, the experiments with E6 further support the hypothesis that induction of p53 by WR1065 is not the consequence of its capacity to alter DNA, either directly or indirectly.

One of the most spectacular intracellular changes in MCF-7 cells exposed to WR1065 is the marked 2-fold increase in the level of GSH (Fig. 2). It has been reported that WR1065 has complex interactions with intracellular glutathione metabolism [43]. An increase in GSH level has been described by Grdina *et al.*, who have proposed that this mechanism may play a key role in cytoprotection [44]. Although the exact molecular mechanism involved is not known, our observation further substantiates that WR1065 increases the intracellular availability of reducing equivalents. This increase may, in turn affect the activity of transcription factors such as p53. This increase in reducing capacity, together with the supply of thiol equivalent directly provided by WR1065, may result in a form of intracellular ‘‘reducing stress’’. It is tempting to speculate that p53 may become activated as a part of a cellular pathway of response to such a form of stress. A good candidate as effector in transducing these stress signals to p53 is the c-Jun N-terminal kinase (JNK/SAPK), which has been shown to be activated in response to anti-oxidants [45,46]. This hypothesis is consistent with our recent observation that a dominant-negative mutant form of JNK at least partially inhibits p53 activation by WR1065 [42]. Moreover, recent evidence indicate that glutathion-S-transferase P1-1 may act as a critical JNK-binding protein which inhibits its kinase activity [47]. This interconnection may contribute to explain the effects of WR1065 on JNK.

Further work is required to determine the contribution of p53 activation to cytoprotection. It unlikely that cytoprotection by WR1065 may be explained only in terms of effects on p53. However, previous data from our group suggest that WR1065 has a cytostatic effect on p53-competent, but not on p53-deficient cell lines ([21], unpublished results). This hypothesis is consistent with results showing that activation of p53 can indeed protect some cell types from undergoing delayed p53-independent apoptosis

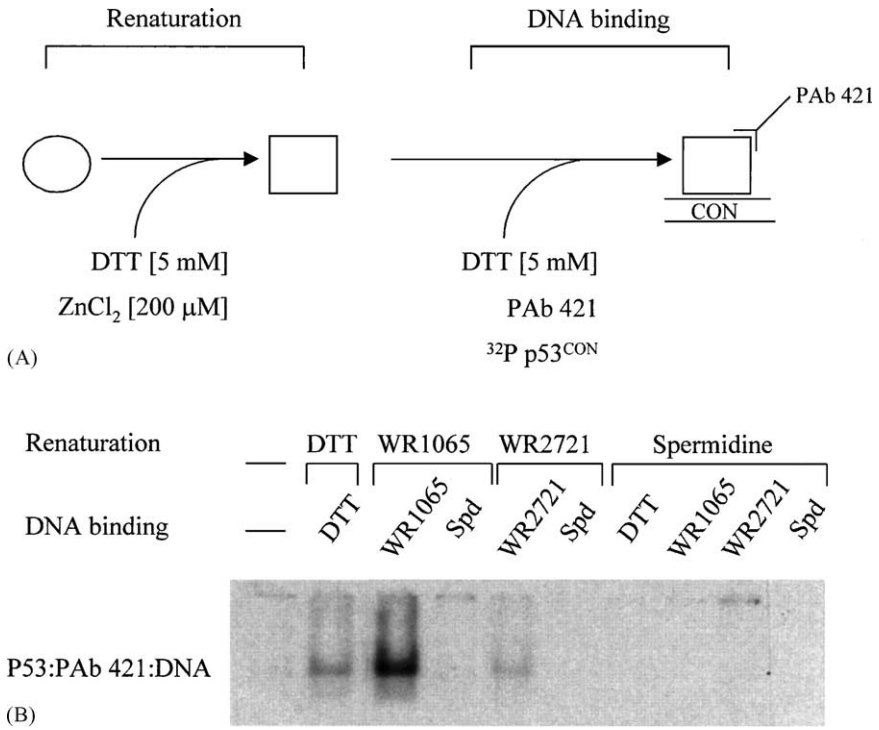

Fig. 5. WR1065 mediates renaturation of recombinant wild-type p53 and stimulates DNA-binding *in vitro*. Protein extracts of S*f*-9 cells expressing human wild-type p53 were prepared in the presence of 5 mM DTT and 200 μM EDTA. Equal amounts of protein (25 μg) were incubated for 20 min with either DTT, WR1065, WR2721 or spermidine at 5 mM, in the presence of 200 μM $ZnCl_2$. DNA-binding experiments were performed using the ^{32}P-labeled oligonucleotide $p53^{con}$, in the presence of PAb 421 (which stabilizes and supershifts p53:DNA complexes, see Section 2). (A) Principle of the two-steps renaturation and DNA-binding assays. p53 conformation are depicted by a circle (inactive conformation, unable to bind DNA) and a square (fully folded conformation, competent for DNA-binding). CON: consensus DNA sequence. (B) DNA-binding activity as detected by EMSA. Only the portion of the autoradiogram with the specific p53:PAb 421:DNA complexes is shown.

in response to various forms of DNA damage, by temporarily removing them from the pool of cells capable of replicating DNA [48]. Such an effect may contribute to afford a degree of protection to cells expressing wild-type p53, as compared to p53-deficient cells. We thus propose that p53 activation by WR1065 through redox-mechanism and DNA-damage-independent pathway may represent an important step in the mechanism of action of WR1065.

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