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Binding of the Aminothiol WR-1065 to Transcription Factors Influences Cellular Response to Anticancer Drugs

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ABSTRACT

The aminothiol WR-1065 (the active form of amifostine) protects normal tissues from the toxic effects of certain cancer drugs, while leaving their antitumor effects unchanged. The present data address the mechanism of action of this dichotomous effect. ^{35}S-Labeled WR-1065 bound directly to the transcription factors nuclear factor-κB, activator protein-1, and p53, resulting in enhanced binding of these proteins to target regulatory DNA sequences and subsequent transactivation of a number of downstream genes. Since other small molecular thiols could mimic WR-1065, the redox potential of the sulfhydryl is an important determinant of its activity. In nontransformed cells, WR-1065 protected cells from the cytotoxic effects of paclitaxel in a p53-dependent manner. However, in a transformed human tumor cell line, there was no cytoprotectivity by WR-1065, consistent with the premise that p53-dependent growth arrest is the basis for the protective effect of this compound, and that this pathway is abrogated in human tumors. The combined data support the principle that the cellular effects of the aminothiol WR-1065 are mediated through an impact on transcriptional regulation and are not only a consequence of radical scavenging.

WR-1065 is the active form of amifostine (Ethyol, WR-2721), a phosphorylated aminothiol prodrug. Amifostine is generally considered to be dephosphorylated at the tissue site by membrane-bound alkaline phosphatase to WR-1065, which is subsequently taken up into cells (Capizzi, 1999). A major pharmacological benefit of amifostine ensues from its dual effects: 1) amifostine protects normal tissues from the toxic effects of ionizing radiation and chemotherapeutic agents, and 2) amifostine leaves the antitumor effects of these agents either unchanged or enhanced (van der Vijgh and Peters, 1994; Capizzi, 1999). In a series of randomized clinical trials, administration of amifostine prior to radiation therapy for advanced rectal cancer reduced radiation toxicity, while maintaining the therapeutic benefits of treatment (Liu et al., 1992). In trials where cyclophosphamide and cisplatin were administered with and without amifostine, patients with amifostine had significantly fewer toxic effects and no difference in tumor response or survival (Kemp et al., 1996). A recent study reported that combinations of paclitaxel with amifostine protected normal lung fibroblasts from paclitaxel cytotoxic effects, while enhanced cytotoxic effects were achieved in nonsmall cell lung cancer (Taylor et al., 1997). Additionally, amifostine sensitized leukemic stem cells to the cytotoxic effect of mafosfamide, while normal marrow progenitor cells were protected from cytotoxicity (Douay et al., 1995).

The cytoprotective benefits of amifostine are thought to be mediated by the nucleophilicity of the thiol moiety. Preferential protection of normal cells has been speculated to occur either because of the higher activity of membrane-bound alkaline phosphatase in normal cells, and/or because of pH differences between normal and tumor cells, which would alter alkaline phosphatase activity. However, the recent observation that WR-1065 protects normal human diploid fibroblasts from paclitaxel toxicity, while fibrosarcoma cells were unaffected or sensitized, suggests a less straightforward explanation for the therapeutic effect of this drug (Zhang et al., 1992).

NF-κB, AP-1, and p53 are transcription factors that are known to be highly sensitive to redox status in the cell, and to participate in the decisions between cell proliferation and apoptosis (Arrigo, 1999; Gius et al., 1999; Kamata and Hirata, 1999). NF-κB is a heterodimer of p50 and p65 and is

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ABBREVIATIONS: NF-κB, nuclear factor-κB; AP-1, activator protein-1; MEF, murine embryo fibroblast; wt, wild type; PBS, phosphate-buffered saline; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; PAGE, polyacrylamide gel electrophoresis; GSH, glutathione; GSH-MEE, glutathione monoethyl ester; TLK117, γ-glutamyl-*S*-(benzyl)cysteinyl-*R*-phenyl glycine.

sequestered within the cytosol by association with an inhibitory protein, IκB (Karin and Smeal, 1992). The activation of NF-κB following a stimulus is largely post-translational, and results from the dissociation of the NF-κB:I-κB complex followed by translocation of the released NF-κB into the nucleus (Brockman et al., 1995; Chen et al., 1996), resulting in the subsequent transcriptional activation of target genes. AP-1 is a sequence-specific transcription factor composed of either homo- or heterodimers between members of the c-Jun and c-Fos families (Kerppola and Curran, 1995). The exact mechanisms that regulate the assembly, targeting, and functional specificity of these proteins remain unclear, although post-translational modification, altered DNA-binding activity, conformational changes, and altered gene expression have been suggested (Karin and Hunter, 1995). It is well documented that p53 is an important regulator of cell growth and death (Meek, 1999). Following exposure to various stress stimuli, p53 induces cell cycle arrest to prevent the replication of damaged DNA or apoptosis by regulating downstream genes to eliminate defective cells (Levine, 1997). In the majority of human tumors, p53 is mutated or inactivated through association with other regulatory proteins (Levine, 1997). Therefore, like NF-κB and AP-1, activation of p53 can mediate the cellular decision between cell growth, proliferation, and death (Jimenez et al., 1999).

In the present study, the effects of the aminothiol WR-1065 on the activities of NF-κB, AP-1, and p53, all of which are known to be sensitive to redox changes in the cell, were investigated. WR-1065 activated these three transcription factors in the cell and induced up-regulation of their target genes. Furthermore, in combination with paclitaxel, WR-1065 protected normal fibroblasts from cytotoxicity of paclitaxel in a p53-dependent manner, while the antitumor activity in human melanoma cells was enhanced. These results provide a molecular basis for the diverse effects of WR-1065.

Materials and Methods

Cell Culture and Cytotoxicity Assays. The p53-null murine embryo fibroblast (MEF) cell line 10.1, and the wild-type (wt) p53 MEF cell line 12.1 were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate. The CaCl cells are human melanoma cells with wild-type p53, while the CaCl/E6 cells stably express the human papillomavirus 16 E6 protein, which targets p53 for degradation. The CaCl and the CaCl/E6 cells were grown in the same media as described above, with the latter being grown in the presence of 700 μg/ml G418 sulfate to maintain the E6 construct. To test the effects of paclitaxel in the presence or absence of WR-1065 on cell growth, cells were seeded in 96-well tissue culture dishes at 20% confluence and were allowed to attach and recover for at least 24 h. Varying combinations of paclitaxel alone or in combination with a 60-min pretreatment with 1 mM WR-1065 were then added to each well, and the plates were incubated for an additional 48 h (HeLa cells) or 72 h (all other cell lines analyzed). The number of surviving cells was determined by staining with sulforhodamine B as described (Skehan et al., 1990). The percentage of cells killed by paclitaxel and/or WR-1065 was calculated as the percentage decrease in sulforhodamine B binding compared with control cells. Control cells had equal amounts of ethanol and/or phosphate-buffered saline (PBS) added to them.

Preparation of Nuclear Extracts. Nuclear extracts were prepared as described (Dignam et al., 1983). HeLa cells were washed with ice-cold PBS, scraped, and collected by centrifugation. Cell pellets were resuspended in three packed cell pellet volumes of ice-cold buffer A (10 mM HEPES, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; 0.1 mM EDTA; protease inhibitors) and kept on ice for 10 min. The cells were then lysed by homogenization and the homogenates were centrifuged for 15 min at 3300*g*. The pellets were resuspended in 2 volumes of buffer B (20 mM HEPES, pH 7.9; 25% glycerol; 0.4 M NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; protease inhibitors). The suspension was stirred gently with a magnetic stirring bar for 30 min and then centrifuged at 25,000*g*. The supernatants were dialyzed against 100 volumes of buffer C (20 mM HEPES, pH 7.9; 20% glycerol; 0.1 M KCl; 0.2 mM EDTA; protease inhibitors) for 6 h with two changes of buffer C and centrifuged at 3000*g* for 5 min. The resulting supernatants were stored at −80°C until used. Dithiothreitol (DTT) was omitted from all buffers.

Electrophoretic Mobility Shift Assay (EMSA). Assays were carried out using the Promega (Madison, WI) gel shift assay system. The oligonucleotides (NF-κB: 5′ AGT TGA GGG GAC TTT CCC AGG C 3′; AP-1: 5′ CGC TTG ATG AGT CAG CCG GAA 3′) were end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Binding reactions were carried out in a total volume of 10 μl containing: 10 μg of nuclear protein; 10 mM Tris-HCl, pH 7.5; 4% glycerol; 1 mM $MgCl_2$; 0.5 mM EDTA; 50 mM NaCl; 0.05 mg/ml poly(dI-dC)·poly(dI-dC). The reactions were incubated at room temperature for 10 min, and then 1 μl (50,000 cpm) of ^{32}P-labeled oligonucleotides was added followed by an additional 20 min and electrophoresed on an 8% nondenaturing polyacrylamide gel. The gel was dried and exposed to X-ray film.

Reporter Assays. Assays were performed using Promega PathDetect In Vivo Signal Transduction Pathway *cis*-Reporting Systems. The luciferase expression vectors under the control of synthetic promoters that contain the binding site of NF-κB or AP-1 were cotransfected with pSV-β-galactosidase vector into HeLa cells using LipofectAMINE (Invitrogen/Life Technologies Inc., Grand Island, NY). After a 48-h incubation, the media was replaced with fresh media containing drugs and further incubated for 5 h. Cell lysates were prepared using Promega Reporter Lysis buffer. Luciferase and β-galactosidase activity were measured according to the manufacturer's instructions.

Immunoprecipitation. One milligram of whole cell lysate was incubated with 50 μCi of [^{35}S]WR-1065 (custom synthesized by PerkinElmer Life Science Products, Boston, MA) in 100 mM HEPES, pH 7.5 for 15 min at room temperature. This lysate was precipitated with a polyclonal anti-p53 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and washed as described previously (Shen et al., 1999). Following separation on nonreducing SDS-PAGE, the radioactivity was assessed using a Fuji PhosphoImager.

Northern and Western Analyses. Total RNA was isolated from cells using CsCl purification (Murphy et al., 1999) or using TRIzol, as per the manufacturer (Life Technologies, Grand Island, NY). Northern analyses were performed as described (Murphy et al., 1999). Probes for Northerns were radiolabeled using random primers (Prime-It-II; Stratagene, La Jolla, CA) and [α-^{32}P]dCTP (PerkinElmer Life Science Products). Autoradiographs were quantitated using NIH Image software. For Western analyses, cells were treated with 1 mM WR-1065 for 24 h, and subconfluent cultures of cells were harvested and lysed in RIPA buffer (50 mM Tris pH 7.4/150 mM NaCl/1% Triton X-100/0.1% SDS/1% sodium deoxycholate) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, and 5 μg/ml leupeptin). Protein concentrations were determined by a detergent-compatible assay (Bio-Rad DC assay; Bio-Rad, Hercules, CA). Western blots were blocked and incubated in antibody in PBS/0.2% Tween 20/5% nonfat dry milk. Blots were incubated with 1 μg/ml antibody for 1 h at room temperature, followed by washing in PBS/0.2% Tween 20 and incubation in peroxidase-conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) and chemiluminescence detection (PerkinElmer Life Science Products).

Results

WR-1065 Enhances DNA Binding Activity of NF-κB and AP-1. To investigate the effect of WR-1065 on DNA-binding activity of transcription factors NF-κB and AP-1, the EMSA was used, with nuclear extracts prepared with the omission of DTT. As shown in Fig. 1, A and B, preincubation of nuclear extracts with 1 mM WR-1065 for 30 min resulted in enhanced DNA-binding activity of both NF-κB and AP-1. The specificity of this binding was confirmed using competition assays with excess unlabeled oligonucleotides of NF-κB or AP-1, which competed off specific binding, while nonspecific competitor oligonucleotides (Sp1) had no effect (Fig. 1), confirming that the binding is specific. Consistent with the results reported by others (Toledano and Leonard, 1991; Das et al., 1995), reducing agents such as DTT also enhanced the DNA binding of both transcription factors. The DNA-binding activity was increased in a WR-1065 concentration-dependent manner (Fig. 1, C and D).

To ascertain the importance of the thiol moiety of WR-1065 for the activation of DNA binding by NF-κB and AP-1, other reagents were tested using the EMSA assay. As shown in Fig. 1, E and F, reduced glutathione (GSH) also enhanced the DNA binding of NF-κB and AP-1. To assess whether the peptide backbone of GSH had an impact on DNA binding, γ-glutamyl-*S*-(benzyl)cysteinyl-*R*-phenyl glycine (TLK117, a GSH peptidomimetic lacking a free thiol group), azidophenacyl-glutathione (a GSH conjugate lacking a free thiol group) (Shen et al., 1997, 1999) oxidized glutathione, and WR-33278, a disulfide form of WR-1065, were also examined. These four agents had no effect on the DNA-binding activity of NF-κB and AP-1. The combined data indicate that the free thiol of WR-1065 is a critical requirement for the ability of this agent to enhance the DNA-binding activity of the transcription factors NF-κB and AP-1.

WR-1065 Binds to the p50 Subunit of NF-κB and c-Jun Subunit of AP-1. To ascertain whether the enhancing effect of WR-1065 on the DNA-binding activity of NF-κB and AP-1 was due to increased expression of these proteins, immunoblot analyses were carried out. Treatment of HeLa cells for 0, 2, 8, and 24 h with 1 mM WR-1065 did not change the quantitative levels of the p65 and p50 subunits of NF-κB nor the c-Jun and c-Fos subunits of AP-1 (data not shown). Next, we asked whether the enhanced DNA binding of these proteins in the presence of WR-1065 was due to a direct interaction of this drug with these redox-sensitive transcription factors. To this end, ^{35}S-labeled WR-1065 was used as a radioactive probe, and purified p50 subunit of NF-κB and c-Jun subunit of AP-1 (Promega) were incubated with [^{35}S]WR-1065 and subjected to nonreducing SDS-PAGE. Radiolabeled proteins were detected by autoradiography. As shown in Fig. 2, both the p50 subunit of NF-κB and the c-jun protein were radiolabeled following incubation of these proteins with ^{35}S-labeled WR-1065. Some other minor bands were also apparent. These are most likely multimers or breakdown fragments usually detected under nonreducing SDS-PAGE (Shen et al., 1991). In contrast, when the [^{35}S]WR-1065-labeled protein samples were subjected to reducing SDS-PAGE, ^{35}S labeling of proteins was not detectable (data not shown), indicating that WR-1065 bound to these proteins via disulfide bonds.

Transcriptional Activation of NF-κB and AP-1 Target Genes by WR-1065. To study the effect of WR-1065 on

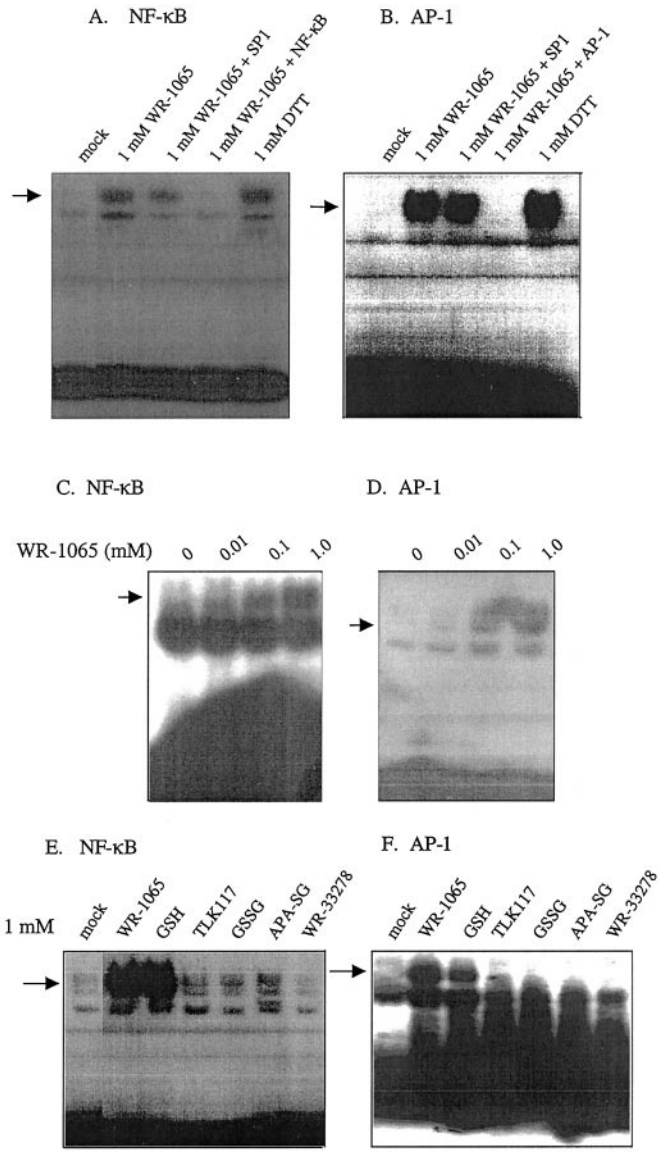

Fig. 1. Effect of WR-1065 on DNA-binding assay for NF-κB and AP-1. Ten micrograms of nuclear extract prepared from HeLa cells was preincubated with WR-1065 for 30 min, and then incubated with ^{32}P-labeled NF-κB (A) or AP-1 (B) probe. The DNA-binding activity of NF-κB (C) or AP-1 (D) was dependent on the WR-1065 concentration. Effects of GSH, GSH analog, or GSH conjugates on the DNA-binding activity of NF-κB (E) or AP-1 (F) were also examined. GSSG, glutathione disulfide; APA-SG, azidophenacyl glutathione.

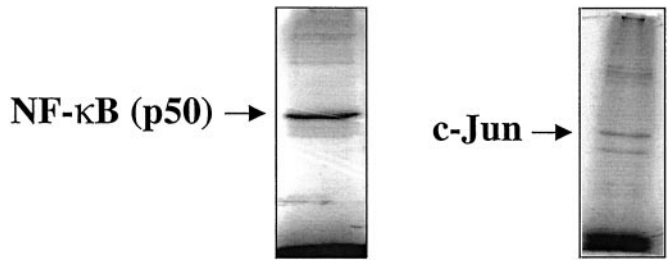

Fig. 2. [^{35}S]WR-1065 binds to NF-κB and AP-1. Purified c-Jun or NF-κB (p50) were labeled with [^{35}S]WR-1065 and loaded onto nonreducing SDS-PAGE. The autoradiography was obtained by exposing the gel to Fuji PhosphoImager.

the activation of NF-κB and AP-1 in vivo, reporter assays were carried out using constructs in which the firefly luciferase gene is placed under the control of a synthetic promoter that contains binding sites for NF-κB or AP-1. These constructs were cotransfected with a β-galactosidase reporter construct, driven by the simian virus-40 early promoter, into HeLa cells. Following transfection, cells were treated with WR-1065, and transcriptional activation was assessed by measuring luciferase activity in these cells (Fig. 3). Significantly, treatment with WR-1065 led to a 3-fold increase in luciferase expression driven by AP-1, and a 5-fold increase when this reporter gene was driven by NF-κB (Fig. 3), when these values were normalized to the level of the cotransfected β-galactosidase gene. In contrast, the luciferase activity of the control luciferase gene was not changed by treatment with WR-1065 (Fig. 3C). Since reduced GSH also enhanced the DNA-binding activity of both transcription factors, glutathione monoethyl ester (GSH-MEE), which is deesterified upon cellular uptake, was used to increase intracellular GSH levels. As shown in Fig. 3, GSH-MEE had no effect on the luciferase activity controlled by NF-κB- and AP-1-binding sites. Paclitaxel alone had no impact on the enzyme activity. The combination of paclitaxel and WR-1065 had a similar effect to that of WR-1065 alone.

WR-1065 Stabilizes p53 Protein and Induces p53-Response Genes HDM2 and p21/waf1. To evaluate the effect of WR-1065 on the p53 tumor suppressor protein, the human tumor cell lines MCF-7 (breast carcinoma) and CaCl (melanoma), both of which express wt p53, were treated with 1 mM WR-1065 for up to 24 h, and p53 expression was examined by Western analysis. In both cell lines, p53 protein levels were increased in a time-dependent manner by WR-1065 treatment, to levels equivalent to those induced by treatment with ultraviolet irradiation (4 J/m^2) (Fig. 4A). Northern blot analysis showed no change in p53 transcript levels following WR-1065, suggesting that the accumulation of p53 protein in WR-1065-treated cells was a post-translational event (data not shown), perhaps due to the change in p53 phosphorylation or redox status of the protein. However, the exact mechanism(s) remains to be clarified. To assess the possibility that WR-1065 activated p53 as a transcription factor, the expression of the p53-response genes HDM2 and p21/waf1 was monitored in MCF-7 cells treated with WR-1065. These studies revealed that WR-1065 enhanced the p53-dependent induction of these two gene products (Fig. 4B). To address the possibility that WR-1065 bound directly with p53, immunoprecipitation analysis was carried out using [^{35}S]WR-1065. For these studies, lysates of Sf9 cells infected with a baculovirus expressing wt p53, or uninfected cells, were incubated with [^{35}S]WR-1065 and immunoprecipitated with p53 antisera. Following fractionation on nonreducing SDS-PAGE, radioactivity was detected by PhosphoImager analysis. As shown in Fig. 5C, [^{35}S]WR-1065-labeled p53 was detected in the lysate from cells infected with p53 baculovirus, but not in uninfected cells, supporting the premise that WR-1065 modifies p53 directly.

WR-1065 Treatment Induces Transactivation of p53-Response Genes in a p53-Dependent Manner. Northern blot analysis of the p53-induced genes bax, KILLER/DR5, fas, gadd45, HDM2, and p21/wafl were performed in an effort to determine the extent to which WR-1065 activated p53 as a transcription factor. These analyses were performed on the human melanoma cell line CaCl, which contains wt p53, as well as on a clonal derivative of these cells (CaCl/E6), which expresses the E6 gene of human papillomavirus 6, which

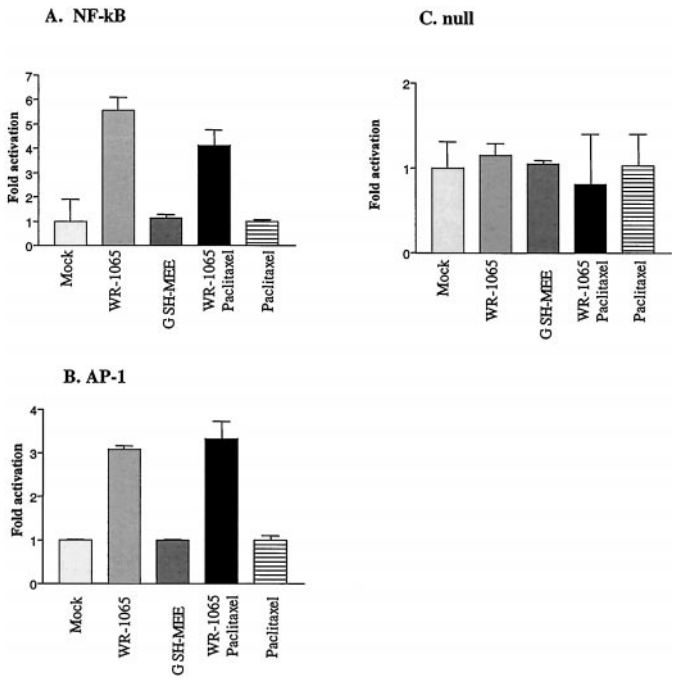

Fig. 3. Transcriptional activation of NF-κB and AP-1 target genes by WR-1065. The luciferase expression vector under the control of synthetic promoters that contain the binding site for NF-κB or AP-1 (PathDetect in Vivo Signal Transduction Pathway *cis*-Reporting Systems; Promega) was cotransfected with pSV-β-galactosidase vector into HeLa cells using transfection reagents. After 48-h incubation, the media were replaced with fresh media containing drugs (1 mM WR-1065, 1 mM GSH-MEE, 10 nM paclitaxel) and further incubated for 5 h. Cell lysates were prepared using Promega Reporter Lysis buffer. Luciferase and β-galactosidase activities were measured and values shown were normalized with respect to transfection efficiency. Null *cis*-reporter plasmids controlled by a basic TATA element were used as control.

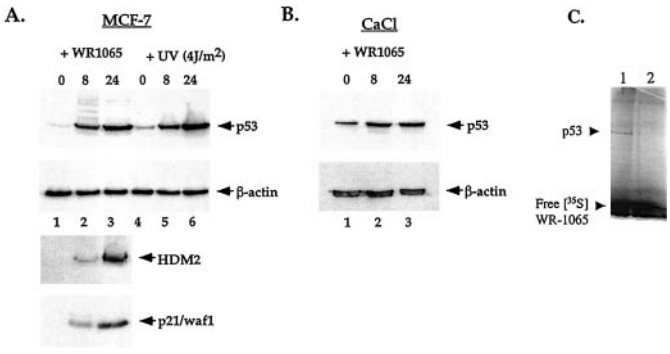

Fig. 4. WR1065 induces an accumulation of wt p53 protein, as well as the p53-response genes p21/waf1 and HDM2. A, Western analysis of MCF7 cells (wt p53) treated with 1 mM WR1065 for the indicated time points. p53 protein is stabilized to levels equivalent to MCF7 cells treated with 4 J/m^2 ultraviolet radiation (compare lanes 2 and 3 to lanes 5 and 6). This induction of p53 protein leads to increased levels of the p53-response genes HDM2 and p21/waf1. β-Actin is included as a control for protein loading. B, Western analysis of CaCl cells (human melanoma, wt p53) treated with 1 mM WR-1065 for the indicated time points. p53 protein is induced approximately 2.5-fold (lane 3). β-Actin is included as a control for protein loading. C, [^{35}S]WR-1065 binds to p53. [^{35}S]WR-1065-labeled cell lysates were immunoprecipitated using anti-p53 polyclonal antibody and separated on nonreducing SDS-PAGE. The autoradiography was obtained by exposing the gel to Fuji PhosphoImager. Lane 1, Sf9 cells infected with wt p53 baculovirus; lane 2 uninfected cells.

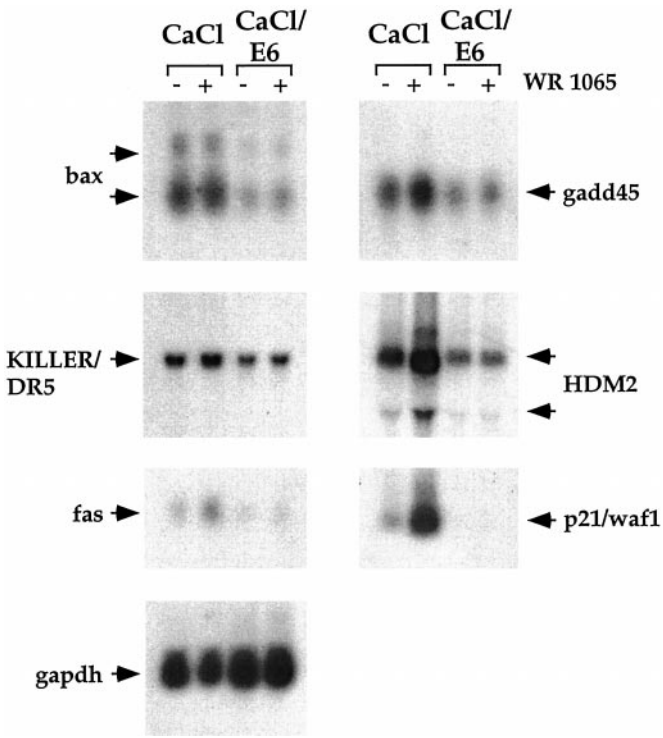

Fig. 5. WR-1065 leads to induction of p53-response genes in a p53-dependent manner. Northern analysis of the p53-reponse genes bax, KILLER/DR5, fas, gadd45, HDM2, and p21/waf1 in CaCl melanoma cells treated with WR-1065, compared with an identically treated clonal derivative of these cells that expresses the human papillomavirus E6 gene, which targets p53 for degradation (CaCl/E6). Cells were treated for 24 h with 1 mM WR-1065 and harvested for RNA isolation. GAPDH is included as a control for RNA loading and integrity.

targets p53 for degradation. Over 90% of the p53 protein is degraded in the CaCl/E6 cell line (Ahn et al., 1999). Northern analysis of CaCl and CaCl/E6 cells treated with 1 mM WR-1065 for 24 h revealed that all of the p53-induced genes analyzed were transactivated following WR-1065 treatment, in a p53-dependent manner. While bax and KILLER/DR5 were induced less than 2-fold, the levels of fas, gadd45, Hdm2, and p21/waf1 were increased from 5- to 20-fold (Fig. 5).

WR-1065 Protects MEFs from Cytotoxic Effects of Paclitaxel in a p53-Dependent Manner, but Has No Effect on Paclitaxel Cytotoxicity in CaCl and CaCl/E6 Tumor Cell Lines. The influence of activating p53 by WR-1065 on the response to cytotoxic drugs was assessed by measuring paclitaxel cytotoxicity in combination with WR-1065 in two sets of matched cell lines that differ in p53 status. The 10.1 and 12.1 cells are nontransformed MEF lines that differ in p53 status (Harvey and Levine, 1991). Similarly, CaCl and CaCl/E6 cell lines are transformed cell lines that differ in p53 status due to expression of human papillomavirus E6 (Ahn et al., 1999). In the absence of WR-1065, the IC_{50} value of paclitaxel in wt p53-expressing MEF cells was not significantly different from p53-null MEF cells (59 versus 54 nM; Table 1). However, in the presence of 1 mM WR-1065, these values were significantly different, with 12.1 cells (wt p53) being protected from paclitaxel by WR-1065 (180 versus 54 nM, in the presence and absence of WR-1065, respectively). This cytoprotective effect appears to require p53, because it did not occur in p53-null fibroblasts, which actually demonstrated enhanced paclitaxel cytotoxicity in the presence of WR-1065 (Table 1). Interestingly, this effect was not recapitulated in transformed cells, which typically are resistant to the effects of p53-dependent growth arrest and apoptosis. Both the CaCl and CaCl/E6 cell lines showed similar paclitaxel cytotoxicity in the presence and absence of WR-1065; such differential cytoprotectivity by WR-1065 in normal and tumor cell lines has been demonstrated previously (Taylor et al., 1997).

TABLE 1

Treatment with WR-1065 protects murine embryo fibroblasts from paclitaxel-induced cell death in a p53-dependent manner

WR-1065 does not protect the human tumor cell line CaCl from the cytotoxic effects of paclitaxel (Taxol). IC_{50} values for paclitaxel were calculated using the sulforhodamine B assay; numbers are in nanomolar (nM) and are the average of three independent experiments ± S.E.M.

Cell Line	IC_{50} Values	
	Paclitaxel Alone	Paclitaxel + 1 mM WR-1065
10.1 (p53 −/− MEF)	59 ± 8	10 ± 5
12.1 (wt p53 MEF)	54 ± 9	180 ± 14
CaCl (wt p53 melanoma)	2.5 ± 0.6	1 ± 0.5
CaCl/E6 (p53 −/− melanoma)	6 ± 0.7	3 ± 0.8

Discussion

The radio and chemoprotective effects of WR-1065 have been ascribed to the nucleophilicity of the sulfhydryl group in the scavenging of reactive oxygen species, and in proton donation during DNA repair reactions (van der Vijgh and Peters, 1994). One of the more interesting aspects of preclinical and clinical amifostine pharmacology relates to studies demonstrating that a therapeutic advantage is achieved by a selective protection of normal tissues when combinations of standard anticancer drugs are used with amifostine (Millar et al., 1982; Valeriote and Tolen, 1982; Douay et al., 1995; Taylor et al., 1997). Since there is no a priori reason to expect differential thiol homeostasis in tumor versus normal tissues, a more specific mechanism(s) for amifostine's effects is implicated. In the current study, we report that the pharmacologically active metabolite of amifostine, WR-1065, activates the DNA-binding activities of NF-κB, AP-1, and p53. All three of these transcription factors are known to be tightly regulated by intracellular redox status (Sen and Packer, 1996; Gius et al., 1999; Kamata and Hirata, 1999). In vitro analysis showed that WR-1065 enhanced the DNA-binding activity of NF-κB and AP-1. While free GSH also showed an enhancing effect on the DNA-binding activity of NF-κB and AP-1, a variety of agents that lack the free thiol moiety had no effect, supporting the importance of this moiety in the activation/binding of WR-1065 to these proteins. The transcriptional activation of NF-κB and AP-1 target genes by treatment of transfected cells with WR-1065 (Fig. 3) confirms that the enhanced DNA-binding activity of both transcription factors in vitro (Fig. 1) translates to a direct impact in living cells.

The molecular mechanism for the activation of p53, NF-κB, and AP-1 almost certainly relies on the ability of this compound to bind directly to these proteins, all of which contain cysteine residues that are sensitive to redox state and critical for activity. Cys 62 residue in the p50 subunit of NF-κB has been reported to be involved in disulfide bond and dimer formation in an oxidative environment. That this cysteine

residue is critical to NF-κB function was demonstrated when its replacement with serine was shown to result in a loss of DNA-binding activity (Matthews et al., 1992). Similarly, the single cysteine residue in a highly conserved peptide (Lys-Cys-Arg) of the c-Jun and c-Fos subunits of AP-1 is also important for the DNA-binding activity of these proteins (Abate et al., 1990). WR-1065 may provide reducing equivalents, thus maintaining these critical cysteine residues in a reactive state. Direct binding of WR-1065 to these proteins may provide a thiol:disulfide exchange reaction, "attaching" the aminothiol in such a way that bulkier proteins that may function as endogenous suppressors are displaced. This activity could serve to enhance DNA binding and transcriptional activation. Our data also suggest that unlike WR-1065, GSH did not stimulate NF-κB- and AP-1 site-driven luciferase transcription. The size of the GSH tripeptide or the difference in nucleophilic selectivity (Pearson and Songstad, 1967) between the cysteine of GSH and the aminothiol of WR-1065 may explain the different biological effects of these sulfhydryl groups.

It was recently reported NF-κB and p53 can cooperate to induce programmed cell death, or apoptosis. Specifically, p53 can induce activation of NF-κB, whereas loss of NF-κB activity was shown to abrogate the ability of p53 to induce cell death, while its ability to induce growth arrest was unaffected (Ryan et al., 2000). The coordinate activation of p53, NF-κB, and AP-1 by WR-1065 demonstrated in this study provides evidence that the ability of WR-1065 to protect normal cells but not tumor cells from the cytotoxic effects of chemotherapeutic agents may rely on its ability to activate the transcriptional response of these transcription factors, and on the differential response of tumor cells to these signaling pathways.

Consistent with a recent report, we found that WR-1065 activates p53 as a transcription factor and leads to increased levels of functional p53 protein (North et al., 2000). Redox sensitivity of p53 has been previously demonstrated (Jayaraman et al., 1997), but this regulation is apparently complicated and may involve regulation of the coordination of zinc between critical cysteine residues (Hainaut and Milner, 1993). Of the 12 cysteine residues in p53, replacement of Cys 173, Cys 235, or Cys 239 with serine was each found to significantly reduce DNA binding by this protein, and completely block transcriptional activation by p53 (Rainwater et al., 1995). Our data show significant direct binding of WR-1065 to p53 and this is hypothesized to contribute protein stability and/or increased DNA-binding activity. Downstream of p53, WR-1065 was found to lead to increased levels of the p53-response genes bax, KILLER/DR5, fas, p21/waf1, and HDM2, indicating that the p53 pathway was activated by this drug. The downstream effect of p53 induction in nontransformed fibroblast lines would be predicted to be growth arrest; our flow cytometry studies of WR-1065-treated MEFs revealed that this drug induces growth arrest in a p53-dependent manner (data not shown). Human tumor cell lines with wt p53 typically inactivate the p53 pathway via deletion of the p53-modifier $p14^{ARF}$, or by amplification of the HDM2 gene, which targets p53 for degradation (Sherr and Weber, 2000). These cell lines would be expected to be more resistant to the effects of p53 than normal cells, and in fact our studies indicate that p53 status has no impact on the ability of WR-1065 to function as a cytoprotective agent. Indeed, a recent report (Kataoka et al., 2000) concluded that radiation protection by WR-1065 in human glioma cell lines was independent of their p53 status. This conclusion is consistent with the principle outlined above, insofar as the downstream p53 pathways may not be operational in these glioma cells. The combined data support the premise that WR-1065 functions as a cytoprotective agent to normal cells via the combined activation of a set of redox-sensitive transcription factors, but activation of these pathways in tumor cells does not lead to growth arrest, and instead leads to preferential killing of tumor cells by cytotoxic agents.

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