

# Amifostine impairs p53-mediated apoptosis of human myeloid leukemia cells

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## Abstract

Amifostine is used as a cytoprotective agent in cancer treatments. Amifostine protects from apoptosis in some models and has been used as hematopoiesis stimulator in myeloid malignancies. As the apoptosis induced by many antitumoral agents is mediated by p53, we studied the effect of amifostine on p53-mediated apoptosis. We used human myeloid leukemia K562 and NB4 cells expressing the temperature-conditional p53-Val<sup>135</sup> mutant. Both cell lines undergo apoptosis at 32°C due to the presence of p53 in wild-type conformation. We found that amifostine dramatically reduced apoptosis by p53 in both cell lines, as assessed by cell morphology, annexin V binding, fraction of sub-G<sub>1</sub> cells, and DNA laddering. To explore the mechanism responsible for this apoptosis protection, we tested the effect of amifostine on p53 transcriptional activity. We found that amifostine reduced p53-mediated transactivation of target promoters in NB4 and K562. Macroarray analysis confirmed that several p53 target genes as *p21<sup>Waf1</sup>*, *mdm2*, *gadd45*, *pig8*, and *pig3* were down-regulated at the mRNA level by amifostine in NB4 and K562. Also, *c-myc* was up-regulated by amifostine in K562 in the presence of p53, consistently with the impairment of p53-mediated apoptosis exerted by c-Myc in these cells. We conclude that amifostine impairs p53-dependent apoptosis of myeloid leukemia cells by reducing the activation of apoptosis-related genes. Our results open the possibility that amifostine could reduce the effectiveness of antitumoral treatments when it is dependent on active p53. (Mol Cancer Ther. 2003;2:893–900)

Received 5/14/03; revised 6/20/03; accepted 6/26/03.

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**Grant support:** Grants PM98-109 and SAF02-4193 from Dirección General de Investigación Científica y Técnica, Spanish Government and grant FIS01-1129 from Spanish Ministerio de Sanidad y Consumo.

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## Introduction

Amifostine (WR-2721, *Ethyol*) is an organic thiophosphate that induces chemoprotection against the toxic effects of radiotherapy and different antineoplastic agents. Amifostine is believed to be dephosphorylated in tissues by membrane-bound alkaline phosphatase to the active form, WR-1065, which is the form that enters the cells (1). Amifostine protects normal tissues from the toxic effects of chemotherapeutic agents and radiotherapy without affecting their antitumor effects. The cytoprotective effect extends to nephrotoxicity, neurotoxicity, and mucositis, and has been used in clinical trials in ovary, cervix, lung, and head and neck cancer (2). The cytoprotective effect of amifostine is thought to be a consequence of its ability to scavenge free radicals (3) and to its antimutagenic effects (4).

It has been reported a beneficial effect of amifostine on acute myeloid leukemia (AML) patients (5) and a correlation with reduction of telomerase activity in AML cells *in vivo* (6). Also, it has been shown that amifostine reduces toxicity on hematopoietic progenitor cells and shortens the engraftment period after autologous bone marrow transplantation (7, 8). *In vitro*, amifostine promotes cell survival, delays apoptosis, and stimulates the growth of colony-forming units from bone marrow cells (9, 10). Thus, amifostine has been used in patients with myelodysplastic syndrome, where it stimulates hematopoiesis, inducing a partial hematopoietic recovery (9, 11). This is consistent with previous work suggesting that the inefficient hematopoiesis in myelodysplastic syndromes is due to an increased cell death in bone marrow precursors (12, 13).

*TP53* is the tumor suppressor gene most frequently inactivated in human cancer, and wild-type p53 induces apoptosis in response to genotoxic stress (14, 15). There is compelling evidence that the apoptotic response of cells to chemotherapy is mediated by wild-type p53, and in many human cancers, p53 inactivation correlates with tumor progression, resistance to therapy, and shortened survival. This correlation is particularly strong in leukemia, where the frequency of *TP53* alterations increases dramatically with tumor progression (16–18). For example, in myelodysplastic syndromes, the incidence of p53 anomalies at diagnosis is undetectable but reaches 20–30% after leukemic transformation (16, 17) and in chronic myeloid leukemia (CML), alterations of p53 are very rare in the chronic phase, but they can be found at frequencies of 10–25% in the blast crisis of the disease (19–21).

The role of p53 on amifostine action is controversial and appears to depend on the cell type. For example, in glioma cell lines, the cytoprotection from radiation mediated by amifostine is independent of p53 status (22), whereas amifostine protects fibroblasts from paclitaxel-induced

apoptosis in a p53-dependent manner (23). On the other hand, it has been shown that WR1065 (the active form of amifostine) induces wild-type p53 accumulation in a breast cancer cell line by a mechanism that involves c-Jun N-terminal kinase activation (24).

However, it is not known whether amifostine directly affects p53-induced cell death. In the present work, we have asked whether amifostine has an effect on p53 function in genetically defined leukemia models. Our results indicate that amifostine protects from apoptosis induced by wild-type p53 in myeloid leukemia cells and that impairs the p53-mediated transactivation of several well-known p53 target genes. Thus, these results suggest that, in some instances, amifostine can impair the response of anticancer treatment.

## Materials and Methods

### Cell Culture and Treatments

Cells were grown in RPMI 1640 supplemented with 10% FCS. Kp53A1 is a K562 derivative (25, 26) and NB4-tsp53 is a NB4 derivative (27). Both lines were transfected with the eukaryotic expression vector pLTRp53cG(val135) carrying the cDNA for a murine temperature-sensitive mutant form of p53 (28). Cells were incubated at 37°C unless otherwise indicated. Amifostine (WR-2721, *Ethyol*, kindly provided by Shering-Plough S.A., Madrid, Spain) was added to growing cultures with a cell density of 250,000 cells per milliliter in all experiments. Cell growth was determined with a hemocytometer and cell viability by trypan blue exclusion test.

### Apoptosis Determinations

The percentage of apoptotic cells was analyzed by May-Grünwald-Giemsa staining of cytocentrifuge preparations. The apoptotic cells were identified by chromatin condensation and marginalization, and cytoplasmic shrinkage. At least 200 cells per sample were analyzed. The internucleosomal fragmentation (DNA laddering assay) was determined as described (26). Binding of annexin V-FITC to cell surface was analyzed by flow cytometry following the manufacturer's instructions (Genzyme Diagnostics, Cambridge, MA). To determine DNA content by flow cytometry, cells were fixed in 90% ethanol and stained with 10 µg/ml propidium iodide in the presence of 200 µg/ml RNaseA. Cytometric analysis was carried out in a FACScan flow cytometer (Becton Dickinson, San Diego, CA) using the CellQuest software.

### Immunoblot and Immunofluorescence

Cell pellets were lysed in a solution containing 150 mM NaCl, 50 mM Tris (pH 8), 20 mM NaF, 1% NP40, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, and 10 µg/ml leupeptin, for 20 min on ice. Samples with 80 µg of protein per lane were separated in polyacrylamide gels and transferred to nitrocellulose membranes (Millipore, Madrid, Spain), using an electroblotter (Bio-Rad, Hercules, CA). Antibodies used were: anti-p53 monoclonal antibody FL-393, anti-poly(ADP-ribose)polymerase (anti-PARP) rabbit polyclonal H250 antibody (both from Santa Cruz Biotechnology, Santa

Cruz, CA), anti-Bcl-2 mouse monoclonal antibody, anti-Bcl-X rabbit polyclonal (both from Transduction Laboratories, San Diego, CA), and anti- $\alpha$ -tubulin rabbit polyclonal antibody (a gift from N. Cowan, New York University, New York). Immunocomplexes were detected by chemiluminescent method (ECL, Amersham Biosciences, Buckinghamshire). Immunofluorescence was performed by standard procedures, using anti-p53 monoclonal antibody. Cell nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI).

### Luciferase Assays

Electroporation of Kp53A1 cells (260 V, 1 mFa) was carried out in a Bio-Rad pulser apparatus. The transfected plasmids were: PG13-P4-luc [luciferase reporter carrying a p53 binding site (29)]; p21<sup>Waf1</sup>-luc (26); and pCMV16-E6 (encoding the papillomavirus E6 gene) (26). Kp53A1 cells ( $2 \times 10^6$ ) were transfected with the promoter reporter plasmids (6 µg) and expression vectors for papillomavirus E6 protein (18 µg) as indicated in the figures. The luciferase activity of cell lysates was carried out with the Dual Luciferase Assay System (Promega, Madison, WI) as described by the manufacturer. One microgram of pRL-TK plasmid (Promega) encoding for *Renilla* luciferase was co-transfected in each case. Promoter activity was defined as the ratio between the firefly and *Renilla* luciferases activities, giving the one unit value to the activity of the promoter at 37°C in the absence of amifostine.

### Northern and Macroarray Analysis

Total RNA was isolated from cells by the acid guanidine thiocyanate method. Northern hybridizations were performed as described (26). Probes for p21<sup>Waf1</sup> and *c-myc* were as described (26). Probe for *mdm2* was a gift from S. Lain (University of Dundee, United Kingdom). To characterize the expression profile of p53-related genes in response to amifostine, the Human p53 Gene Array (SuperArray Bioscience Corp., Frederick, MD) was used. In addition to  $\beta$ -actin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control genes, the macroarray contains cDNA probes from 23 genes involved in p53-induced growth arrest and apoptosis, in duplicated spots. Ten micrograms of total RNA from NB4-tsp53 and Kp53A1 cells incubated at 37°C or 32°C for 8 h in the presence or absence of 1 mM amifostine were converted to cDNA by reverse transcription (M-MLV RT, Invitrogen, Carlsbad, CA), in the presence of [ $\alpha$ -<sup>32</sup>P]dCTP. <sup>32</sup>P-labeled cDNAs were hybridized to the membranes following manufacturer's instructions. The expression of genes was quantified by measuring the radioactivity of the filters with a Molecular Imager apparatus (Bio-Rad). Medium value from each pair of cDNAs was normalized to the medium value from GAPDH signals in the corresponding filter (six spots). pUC18 signals served as background hybridization control in these experiments.

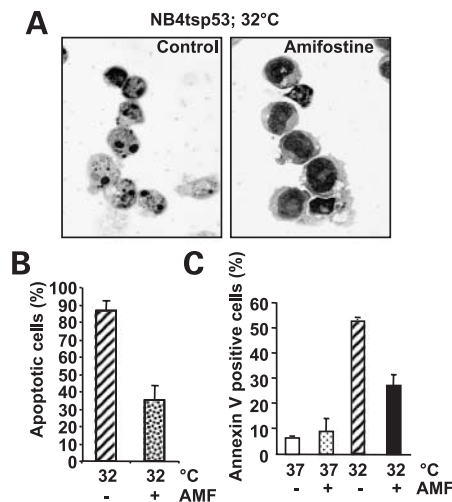
## Results

### Amifostine Impairs p53-Dependent Apoptosis

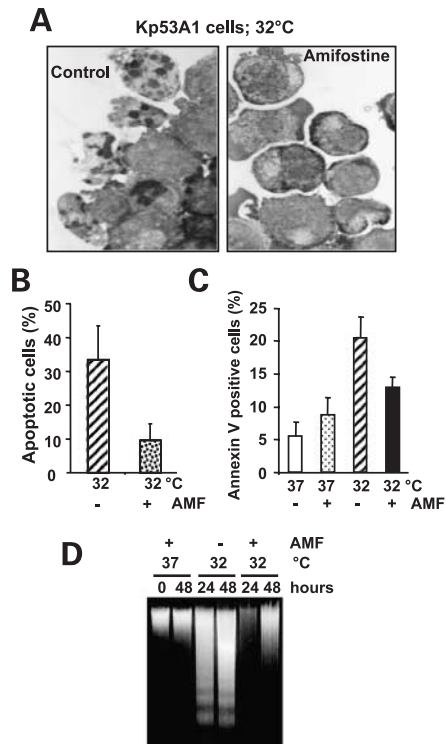
We asked whether amifostine was able to protect human myeloid leukemia cells from p53-dependent

apoptosis. To directly investigate this, we used p53-null cell lines transfected with the murine  $p53^{Val135}$  gene. This is a p53-thermosensitive mutant that adopts a wild-type conformation when shifted to 32°C and exerts the biochemical and biological activities of wild-type p53 (28, 30). We first used NB4-tsp53 cells. These are NB4 expressing  $p53^{Val135}$  (27) and derive from human acute promyelocytic leukemia (M3 subtype of AML). As reported, we observed that the transfer of NB4-tsp53 cells to 32°C resulted in massive apoptosis, reaching almost 90% of the cells showing apoptotic morphology after 24 h at 32°C (Fig. 1A). We analyzed the effect of amifostine on this apoptosis. When the shift to 32°C was done in the presence of 1 mM amifostine, the number of apoptotic cells was reduced by half (Fig. 1, A and B). Apoptosis protection was further confirmed by DNA laddering assay (not shown). The annexin V binding assay that measures the exposure of phosphatidylserine on the outer leaflet of the plasma membrane of apoptotic cells was also reduced by 50% (Fig. 1C). These data showed that the apoptosis induced by p53 in NB4 cells was reduced by amifostine treatment.

We next asked whether amifostine also protected other leukemic cells from p53-mediated apoptosis using Kp53A1 cells. These cells are K562 expressing  $p53^{Val135}$  (25, 26), and derive from human CML. Kp53A1 cells die from apoptosis after shifting to 32°C, a process accompanied by activation of the transcriptional activity of p53 (26). We compared the extent of apoptosis of Kp53A1 cells at 32°C in the presence and absence of 1 mM amifostine. As expected, incubation at 32°C induced apoptosis of Kp53A1 cells, but 1 mM amifostine caused a clear



**Figure 1.** Amifostine protects NB4 cells from p53-mediated apoptosis. **A**, NB4-tsp53 cells were incubated for 24 h at 32°C in the absence (*Control*) or presence of 1 mM amifostine and cyto centrifuge preparations were stained with May-Grünwald-Giemsa. **B**, percentage of apoptotic cells analyzed by May-Grünwald-Giemsa staining. At least 200 cells per experiment were scored. **C**, apoptosis determined by annexin V binding in NB4-tsp53 cells incubated for 24 h at 37°C or 32°C in the absence or the presence of 1 mM amifostine (*AMF*) as indicated. **Bars**, SD from the mean of three independent experiments.

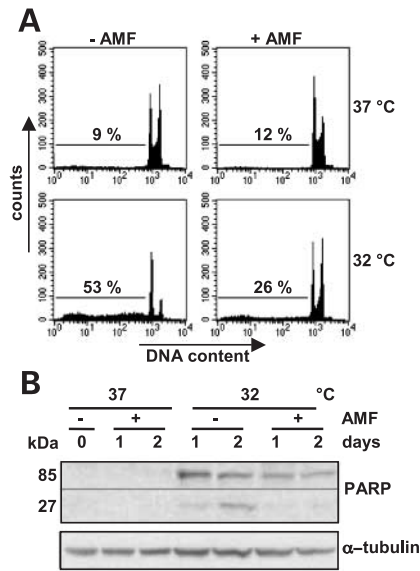


**Figure 2.** Amifostine protects K562 cells from p53-mediated apoptosis. **A**, Kp53A1 cells were incubated for 24 h at 32°C in the absence (*Control*) or presence of 1 mM amifostine and cyto centrifuge preparations were stained with May-Grünwald-Giemsa. **B**, percentage of apoptotic cells analyzed by May-Grünwald-Giemsa staining. At least 200 cells per experiment were scored. **Bars**, SD from the mean of three independent experiments. **C**, apoptosis determined by annexin V binding in Kp53A1 cells incubated for 24 h at 37°C or 32°C in the absence or presence of 1 mM amifostine (*AMF*) as indicated. **Bars**, SD from the mean of three independent experiments. **D**, apoptosis determination by DNA laddering assay. Kp53A1 cells were incubated for the indicated periods of time at 37°C or 32°C in the absence or presence of 1 mM amifostine.

reduction in the number of cells showing apoptotic morphology (Fig. 2, A and B). The extent of apoptosis protection by amifostine was also determined by binding of annexin V to cell surfaces. The quantification by flow cytometry revealed that 1 mM amifostine treatment resulted in a 40% decrease of annexin V-positive cells incubated at 32°C (Fig. 2C). The antiapoptotic effect of amifostine was further confirmed by DNA laddering assay which detects internucleosomal DNA fragmentation (Fig. 2D). Apoptosis protection was also detected in cells treated with lower amifostine concentrations (up to 0.1 mM) but the effect was reduced as compared to that of 1 mM amifostine (not shown). All these data showed that the apoptosis induced by p53 was clearly reduced by amifostine in K562. Mannitol, which is included in the amifostine vials as excipient, did not exert any effect on apoptosis at concentrations of 1 mM (not shown).

To confirm the apoptosis protection exerted by amifostine, we first analyzed the cells with sub-G<sub>1</sub> content of DNA by flow cytometry. As shown in Fig. 3A, treatment of 1 mM amifostine for 24 h at 37°C





**Figure 3.** Amifostine protects K562 cells from p53-mediated apoptosis. **A**, Kp53A1 cells were incubated for 24 h at 37°C or 32°C in the absence or presence of 1 mM amifostine and apoptosis was measured by scoring the number of hypodiploid cells after flow cytometry of propidium iodide-stained cells. **B**, apoptosis of Kp53A1 cells incubated for the indicated days at 37°C or 32°C in the absence or presence of 1 mM amifostine, by immunoblot analysis of PARP proteolysis. The PARP-derived fragments are indicated at the *left*. The filter was also incubated with an anti- $\alpha$ -tubulin antibody to assess the protein loading among samples.

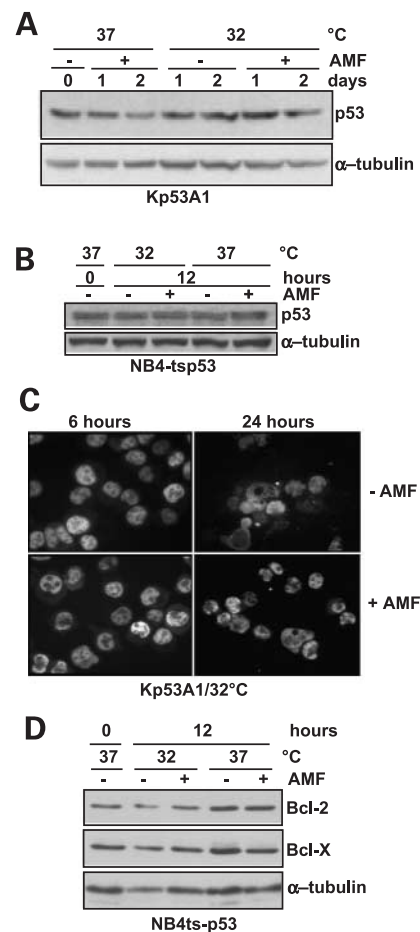
did not provoke significant apoptosis. In contrast, incubation of cells at 32°C and subsequent activation of p53 resulted in a dramatic increase of cells with sub-G<sub>1</sub> DNA content. We found that in cells incubated at 32°C, the fraction of apoptotic cells fell from 53% to 26% by the presence of amifostine. Finally, we also analyzed the effect of amifostine on apoptosis by determining the extent of the caspase-mediated proteolysis of PARP. The immunoblot of Fig. 3B shows that shifting to 32°C resulted in an important proteolysis of PARP, but amifostine decreased the p53-mediated PARP degradation, thus confirming that the drug impairs p53-mediated apoptosis of myeloid cells.

#### Amifostine Does Not Modify p53 Protein Levels or Localization and Does Not Affect Bcl-2 and Bcl-X Expression

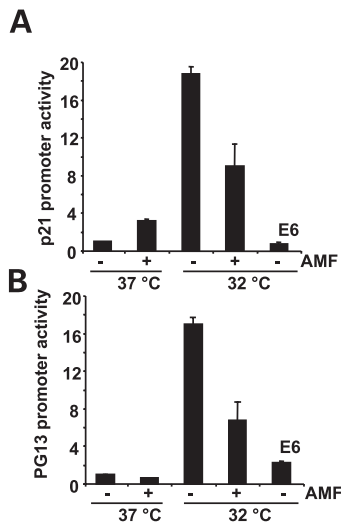
The results described above in two different leukemia cell lines confirmed that amifostine was able to impair p53-mediated apoptosis. In an attempt to elucidate the mechanism by which amifostine exerts this effect, we asked whether amifostine had an effect on p53 protein levels. This is not unprecedented because it has been previously reported that amifostine up-regulates p53 in HCT116 colon cancer cells (31) and MCF7 breast cancer cells by protein stabilization (32, 33). We analyzed p53 by immunoblot and the results demonstrated that amifostine did not provoke significant changes in p53 levels in K562

(Fig. 4A) and NB4 cells (Fig. 4B), while we confirmed the amifostine-mediated accumulation of p53 in MCF7 cells (data not shown). However, it must be noted that Kp53A1 and NB4-tsp53 are transfectant cell lines that express high levels of ectopic p53.

Another possibility was that amifostine provoked a mislocalization of p53. We tested this possibility by immunofluorescence of Kp53A1 cells. The results show that most of the p53 localizes in the nucleus, as expected, and this localization was not modified in cells incubated with amifostine for 6 h (Fig. 4C). After 24 h, p53 localization was also nuclear and the protection of apoptosis by



**Figure 4.** Amifostine does not modify p53 protein levels, p53 localization, and Bcl-2 and Bcl-X expression. Kp53A1 cells (**A**) or NB4-tsp53 cells (**B**) were incubated for the indicated times at 37°C or 32°C in the absence or presence of 1 mM amifostine, lysed, and analyzed by immunoblot for p53 expression. Filters were also incubated with an anti- $\alpha$ -tubulin antibody to assess the protein loading among samples. **C**, localization of p53 in Kp53A1 cells incubated at 32°C for 6 or 24 h in the absence or presence of 1 mM amifostine as indicated. The presence and localization of p53 was analyzed on cytospin preparation of cells by immunofluorescence as described in "Materials and Methods." **D**, NB4-tsp53 cells were incubated for the indicated times at 37°C or 32°C in the absence or presence of 1 mM amifostine, lysed, and analyzed by immunoblot with anti-Bcl-2 or anti-Bcl-X antibodies. Filter was also incubated with an anti- $\alpha$ -tubulin antibody to assess the protein loading among samples.



**Figure 5.** Amifostine impairs p53-mediated transcriptional activation of target promoters. Kp53A1 cells were transfected with p21<sup>Waf1</sup>-luc (A) or PG13-P4-luc (B) p53-target promoter reporter plasmids and expression vector for papillomavirus E6 protein, as indicated. Twenty-four hours after transfection, part of the cells were transferred to 32°C in the absence or presence of 1 mM amifostine and the luciferase activity of the promoters was determined 12 h later. Bars, SD from the mean of three independent experiments.

amifostine was clearly observed (Fig. 4C). A possible mechanism that would explain the amifostine-mediated protection of apoptosis could be the up-regulation of antiapoptotic genes as *bcl-2* or *bcl-x*. However, amifostine did not change Bcl-2 and Bcl-X protein levels in NB4-tsp53 cells at 37°C or 32°C (Fig. 4D).

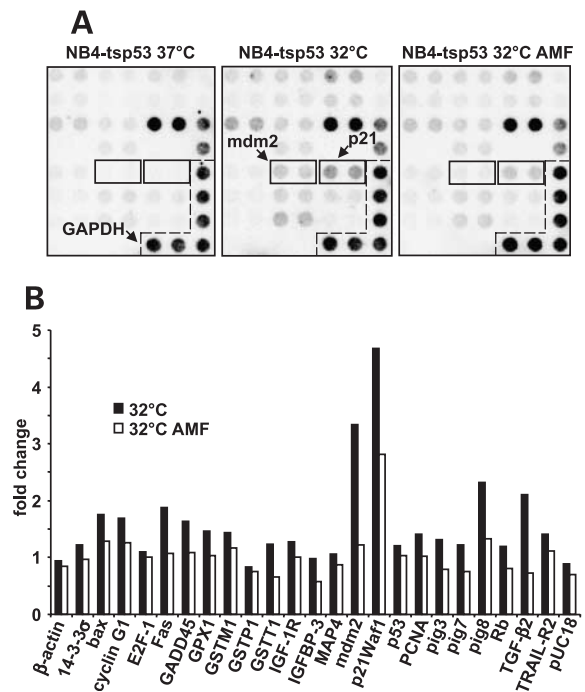
#### Amifostine Impairs Transactivation by p53

To explain the amifostine effect, we asked whether amifostine could impair the transactivation function of wild-type p53 by analyzing the activity of two p53-responsive promoters: p21<sup>Waf1</sup> promoter (Fig. 5A) and the artificial PG13 promoter (29) (Fig. 5B). Kp53A1 cells were transfected with the corresponding luciferase reporter constructs and 24 h after transfection, part of the cells were transferred to 32°C in the absence or presence of 1 mM amifostine. The activity of the promoters was determined 12 h later. As expected, promoter activity was very low at 37°C and dramatically increased after incubation at 32°C (Fig. 5). However, when amifostine was present, the transactivation activity of p53 was significantly impaired. The specificity of the p53 activity on both promoters was confirmed by co-transfection of an expression vector for the papillomavirus E6 gene, which induces p53 degradation. Therefore, amifostine, at the concentrations that impairs p53-mediated apoptosis, also impairs p53-mediated transactivation.

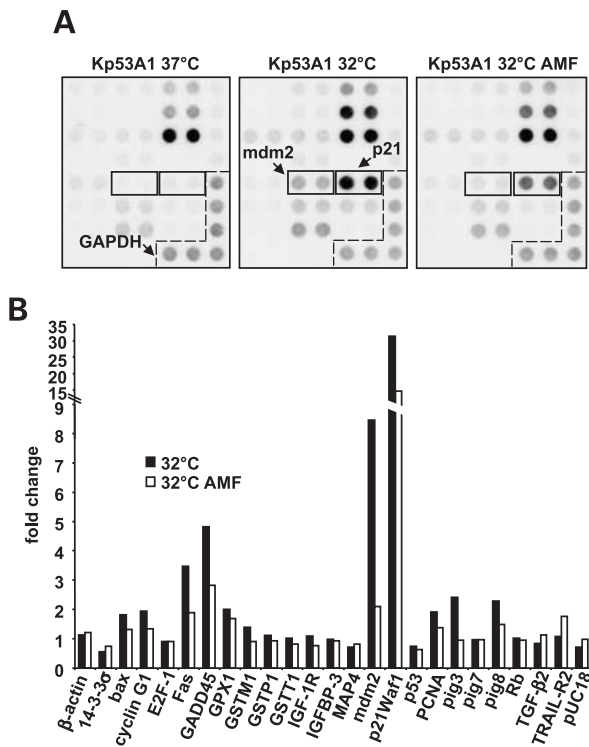
We next asked whether this decrease in transcriptional activity is reflected at the level of p53 target gene steady-state mRNAs. We prepared cDNA samples from NB4-tsp53 incubated at 37°C or 32°C in the presence or absence of 1 mM amifostine for 8 h and hybridized macroarrays with a panel of p53 target genes and other genes related to

genotoxic response (Fig. 6). The results showed the induction of most p53 target genes at 32°C, as expected (compare hybridization results from cells at 37°C versus 32°C) (Fig. 6A). When the effect of amifostine was analyzed, we found that the mRNA levels of several p53 target genes, and particularly p21<sup>Waf1</sup> and *mdm2*, were reduced by the presence of the drug. The signal quantification (Fig. 6B) showed that the extent of this reduction varied with the gene, being maximal for *mdm2* (60%). We next analyzed p53 target genes expression in Kp53A1 under the same experimental design used for NB4-tsp53. The induction of p53 target genes was more prominent than in NB4-tsp53 cells. However, the repressive effect of amifostine was similar in both cell lines, although differences in some genes were also found. The signal quantification indicated the amifostine-mediated reduction in p21<sup>Waf1</sup>, *mdm2*, *gadd45*, *pig3*, and *pig8* mRNAs in Kp53A1 (Fig. 7B). We confirmed the expression changes of p21<sup>Waf1</sup> and *mdm2* by Northern blot hybridization (Fig. 8). Because NB4-tsp53 and Kp53A1 do not express human p53 mRNA, this signal as well as pUC-18 denoted background levels. We conclude that amifostine represses the gene expression activation mediated by p53 and this may lead to defective apoptosis induction in K562 and NB4 cells.

It has been reported that amifostine down-regulates *c-myc* (34). Also, we have previously shown that activation



**Figure 6.** Macroarray analysis of genes regulated by amifostine in NB4 cells. A, human p53 target gene array hybridized with <sup>32</sup>P-labeled cDNA prepared from NB4-tsp53 cells incubated for 8 h at 37°C, 32°C, or 32°C in the presence of 1 mM amifostine as indicated. The localization of GAPDH, p21<sup>Waf1</sup>, and *mdm2* spots in the array are indicated with arrowheads. B, quantification of the signals of macroarray hybridizations. Bars, expression level of each gene normalized to the GAPDH signal as described in "Materials and Methods." The one unit value corresponds to the expression of the gene at 37°C in the absence of amifostine.



**Figure 7.** Macroarray analysis of genes regulated by amifostine in K562 cells. **A**, human p53 target gene array hybridized with  $^{32}\text{P}$ -labeled cDNA prepared from Kp53A1 cells incubated for 8 h at 37°C, 32°C, or 32°C in the presence of 1 mM amifostine as indicated. The localization of *GAPDH*, *p21<sup>Waf1</sup>*, and *mdm2* spots in the array are indicated with arrowheads. **B**, quantification of the signals of macroarray hybridizations. Bars, expression level of each gene normalized to the *GAPDH* signal as described in "Materials and Methods." The one unit value corresponds to the expression of the gene at 37°C in the absence of amifostine.

of p53 in K562 cells results in the repression of *c-myc* expression and that enforced *c-Myc* expression antagonizes p53-dependent apoptosis in K562 (26). Therefore we tested the effect of amifostine on *c-myc* expression in K562 cells in the presence and absence of active p53. The results (Fig. 8) demonstrated that, both amifostine and active p53 (*i.e.*, incubation at 32°C) induced *c-myc* down-regulation. However, the treatment of cells with 1 mM amifostine at 32°C resulted in a significant increase in the *c-myc* mRNA levels.

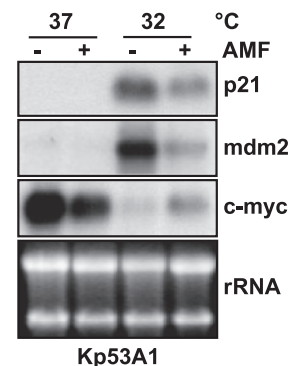
## Discussion

It has been reported that amifostine inhibits apoptosis of human bone marrow precursors (Ref. 8 and our unpublished results with erythroid precursors). The results presented here demonstrate a previously unknown mechanism for the antiapoptotic effect of amifostine in hematopoietic cells, that is, the interference with p53 function. We have directly demonstrated this antiapoptotic effect of amifostine in human myeloid leukemia cell lines K562 and NB4, using transfectants with the thermosensitive mutant p53<sup>Val135</sup>. The parental cells of both lines lack functional

p53 and in the transfectants, the apoptosis after shift to 32°C is a direct consequence of the activity of p53 in wild-type conformation. We showed that amifostine is antagonizing p53-mediated apoptosis in human myeloid leukemia-derived cells (K562 and NB4). It is noteworthy that these cell lines derive from two different types of myeloid leukemia (CML and acute promyelocytic leukemia, respectively). Thus, the impairment of p53-mediated apoptosis by amifostine seems to be a general effect in human myeloid leukemia cells.

Recent reports on MCF7 breast cancer and HCT116 colon cancer cell lines showed that amifostine, at the concentrations used in the present work, stabilizes and up-regulates p53 (23, 31, 32). However, we did not observe such effect in NB4-tsp53, Kp53A1, and murine myeloid 32D cells (which carry wild-type p53), although we confirmed the amifostine-mediated up-regulation of p53 in MCF-7, as assayed by immunoblot (not shown). It has also been suggested that amifostine activates NFκB in myeloid 32D cells, and that this activation may be related to the antiapoptotic mechanism of amifostine (35), but we did not detect amifostine-dependent NFκB activation in K562 cells (not shown). Thus, amifostine exerts different effects on p53 regulation depending on the cell type under study. Taken together, our data in NB4 and K562 cells unambiguously demonstrate that amifostine does not reduce p53 protein levels but impairs p53-mediated apoptosis.

We sought for a mechanism that may explain this effect, and found that p53-dependent activation of target promoters is impaired by the presence of amifostine in K562 and NB4 cells. This effect was confirmed at the mRNA level by macroarray and Northern analysis. Among those genes which p53-mediated up-regulation was impaired by amifostine, we found some involved on cell cycle arrest (*p21<sup>Waf1</sup>*, *gadd45*) and apoptosis induction (*fas*, *mdm2*, *bax*, *pig3*, *pig8*). We previously demonstrated that wild-type p53 down-regulates *c-Myc* in K562 (26) and, consistently, we found in the present work that *c-myc*



**Figure 8.** Expression of *p21<sup>Waf1</sup>*, *mdm2*, and *c-myc* in response to amifostine in the presence or absence of active p53. mRNA expression of the indicated genes in Kp53A1 cells incubated for 8 h at 37°C or 32°C in the absence or presence of 1 mM, as determined by Northern blot. A picture of the filter after transfer showing the rRNAs stained with ethidium bromide is shown to assess the loading and integrity of the RNAs.

is up-regulated by amifostine treatment in K562 expressing wild-type conformation p53. It is of note that c-Myc acts as a survival protein in p53-induced cell death in these cells (26), and therefore the impairment of *c-myc* down-regulation by amifostine helps to explain the antiapoptotic effect of the drug.

It is generally accepted that p53 transcriptional activity is required for efficient induction of apoptosis (36–38). Thus, the data presented here strongly suggest that amifostine antagonizes p53-mediated apoptosis by impairing p53-dependent transcription. The molecular basis for this effect is not clear, but whatever the precise mechanism might be, our results help to explain the protective effect of amifostine in normal hematopoietic precursors and non-tumor cells, which carry wild-type p53. On the other hand, our results open the possibility that this drug can have non-desirable clinical effects. Importantly, the amifostine concentration used in this work is clinically relevant (10, 39). p53 mediates the apoptosis of tumor cells elicited by most anticancer drugs and radiotherapy, and reintroduction of wild-type p53 is the basis of many trials of gene therapy against cancer (40, 41). Thus, suppression of p53 function by amifostine could reduce the effectiveness of therapy, at least in antileukemic treatments. Although it is unknown whether the p53 status modifies the clinical effects of amifostine, our present work suggests that amifostine must be used with caution because it may impair the response to therapy if it is dependent on active p53. Further basic and clinical studies should be conducted to clarify the impact of p53 alterations on the effects of amifostine in cancer.

#### Acknowledgments

We thank Shering-Plough (Spain) for amifostine; Rosalía Martínez, María Aramburu, and Rosa Blanco for technical assistance; Kathleen Cho, Sonia Lain, Abelardo López-Rivas, Moshe Oren, Manuel Serrano, and Bert Vogelstein for plasmids; and Nicholas Cowan for anti-tubulin antibody.

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