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Research Article

Lonidamine, a dichlorinated derivative of indazole-3-carboxylic acid, was shown to play a significant role in reversing or overcoming multidrug resistance. Here, we show that exposure to 50 microg/ml of lonidamine induces apoptosis in adriamycin and nitrosourea-resistant cells (MCF-7 ADR(r) human breast cancer cell line, and LB9 glioblastoma multiform cell line), as demonstrated by sub-G1 peaks in DNA content histograms, condensation of nuclear chromatin, and internucleosomal DNA fragmentation. Moreover, we find that apoptosis is preceded by accumulation of the cells in the G0/G1 phase of the cell cycle. Interestingly, lonidamine fails to activate the apoptotic program in the corresponding sensitive parental cell lines (ADR-sensitive MCF-7 WT, and nitrosourea-sensitive LI cells) even after long exposure times. The evaluation of bcl-2 protein expression suggests that this different effect of lonidamine treatment in drug-resistant and -sensitive cell lines might not simply be due to dissimilar expression levels of bcl-2 protein. To determine whether the lonidamine-induced apoptosis is mediated by p53 protein, we used cells lacking endogenous p53 and overexpressing either wild-type p53 or dominant-negative p53 mutant. We find that apoptosis by lonidamine is independent of the p53 gene.

Lonidamine Induces Apoptosis in Drug-resistant Cells Independently of the *p53* Gene

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Abstract

Lonidamine, a dichlorinated derivative of indazole-3-carboxylic acid, was shown to play a significant role in reversing or overcoming multidrug resistance. Here, we show that exposure to 50 μg/ml of lonidamine induces apoptosis in adriamycin and nitrosourea-resistant cells (MCF-7 ADR^R human breast cancer cell line, and LB9 glioblastoma multiform cell line), as demonstrated by sub-G1 peaks in DNA content histograms, condensation of nuclear chromatin, and internucleosomal DNA fragmentation. Moreover, we find that apoptosis is preceded by accumulation of the cells in the G0/G1 phase of the cell cycle. Interestingly, lonidamine fails to activate the apoptotic program in the corresponding sensitive parental cell lines (ADR-sensitive MCF-7 WT, and nitrosourea-sensitive LI cells) even after long exposure times. The evaluation of bcl-2 protein expression suggests that this different effect of lonidamine treatment in drug-resistant and -sensitive cell lines might not simply be due to dissimilar expression levels of bcl-2 protein.

To determine whether the lonidamine-induced apoptosis is mediated by p53 protein, we used cells lacking endogenous p53 and overexpressing either wild-type p53 or dominant-negative p53 mutant. We find that apoptosis by lonidamine is independent of the *p53* gene. (*J. Clin. Invest.* 1996. 98:1165–1173.) Key words: chemoresistance • breast cancer • glioblastoma • bcl-2 • DNA fragmentation

Introduction

Drug resistance of tumor cells is one of the major obstacles to effective chemotherapy (1). The aim of numerous recent studies has been to identify agents capable of reversing this resistance (2, 3). Lonidamine (LND),[1] a dichlorinated derivative of indazole-3-carboxylic acid, has been shown to revert the resistance of tumor cells to adriamycin (ADR) and *cis*-platinum (4–6). Clinical trials performed in patients with advanced breast cancer have also demonstrated that LND significantly increases the activity of epirubicin (7). LND decreases cell metabolism by reducing ATP production, and alters the cell membrane through its affinity for the inner leaflet of the lipid bilayer (8, 9). These effects are thought to impair tumor cell metabolic adaptations, such as reduction of drug efflux and detoxification, necessary for drug resistance. Indeed, we previously showed that LND overcomes ADR resistance in the human breast cancer cell line MCF-7 ADR^R by increasing intracellular content of the chemotherapeutic agent (4). However, comparing the effects of LND on the cell survival of ADR-resistant and -sensitive cells, we found that with similar intracellular ADR levels, MCF-7 ADR^R cells were much more affected than MCF-7 WT cells (4). Moreover, the intracellular accumulation of ADR induced by LND remained localized in the cytoplasm (10). Thus, modification of intracellular levels of ADR alone is not sufficient to explain the drastic reversal of ADR-resistant phenotype induced by LND.

It has recently been shown that a variety of currently used cancer chemotherapeutic agents exert their effects by inducing apoptotic cell death (11–13). Indeed, the ability of tumor cells to respond to damage and eventually activate the apoptotic program might determine the ultimate success of cancer therapy (14). Apoptosis appears to play a pivotal role in the control of tumor growth by counterbalancing proliferation (15–17). Genes involved in neoplastic transformation, such as *p53* and *bcl-2* are also involved in apoptosis regulation (18), with the *p53* gene being the most frequently altered gene in human cancers (19). Wild-type p53 (wt-p53) protein has been shown to be a mediator of different types of apoptosis, including that induced by several chemotherapeutic agents such as adriamycin, etoposide, and 5-fluorouracil (20). More interestingly, mutations or deletions in the *p53* gene have been associated, in vivo and in vitro, with resistance to apoptosis induced by the same chemotherapeutic agents (14). The *bcl-2* gene is able to confer resistance to apoptosis in several systems (21, 22), and it was found to be overexpressed in follicular lymphomas and leukemias (23, 24). Moreover, expression of *bcl-2* is one potential mechanism by which tumor cells escape p53-mediated apoptosis (25).

Here, we report the results obtained by studying the mechanism(s) through which LND rescues the resistant phenotype of tumor cells. We used two drug-resistant cell lines which differ in histotype and mechanism of drug resistance: ADR-resistant breast cancer cells (MCF-7 ADR^R) and nitrosourea-resistant glioblastoma cells (LB9). We found that LND induces apoptosis in the resistant cells, whereas it does not in the parental-sensitive cells (MCF-7 WT, and LI). In addition, overexpression of mutant-p53 or wt-p53 proteins as well as the use

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1. *Abbreviations used in this paper:* ADR, adriamycin; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; LND, lonidamine; PI, propidium iodide; ts-p53, temperature sensitive p53; vinc, vincristine; wt-p53, wild-type p53.

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of the p53-negative promyelocytic leukemia cell line resistant to vincristine (HL60/Vinc) (26), showed that LND-induced apoptosis is not mediated by p53. These results suggest that LND might be useful in the treatment of drug-resistant tumors presenting p53 alterations (27, 28).

Methods

Cell lines and culture conditions. The breast cancer cell line MCF-7 WT and its ADR-resistant derivative, MCF-7 ADR^R, were kindly provided by Dr. K. Cowan (National Cancer Institute, Bethesda, MD). The LI line was established from surgical specimens of a glioblastoma multiform (29), and its 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU)–resistant derivative (LB9) was kindly provided by Dr. A. Floridi (Regina Elena Cancer Institute, Rome, Italy). The vincristine-resistant derivative HL60 promyelocytic leukemia cell line (HL60/Vinc) was kindly provided by Dr. C. Cucco (Regina Elena Cancer Institute) (30). The tumor lines were maintained in 25 cm^2 tissue culture flasks in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics in a humidified atmosphere with 5% CO_2 at 37°C. MCF-7 ADR^R cells were grown in medium containing 10 μM ADR and passaged for 2 wk in drug-free medium before each experiment.

Lonidamine preparation and treatment. LND (Doridamine; Angelini S.p.a., Rome, Italy) was dissolved in DMSO at a concentration of 10 mg/ml, adjusted with isoton to a 10× concentration, and brought to the final concentration with supplemented medium. Each experiment was performed on exponentially growing cells employing freshly prepared drug solutions. Approximately 5×10^5 cells were plated in 100-mm tissue culture dishes and incubated at 37°C for 3 d before exposure to LND-containing medium. Resistant cells were exposed to 50 μg/ml LND for 12, 24, and 48 h while sensitive parental cells were treated with the same amount of LND for periods of time ranging from 12 to 72 h. Cells floating in the culture supernatant were collected by centrifugation and pooled with adherent cells recovered from the plates by trypsin-EDTA treatment. Cells were washed, assayed for cell viability (trypan blue exclusion test), and counted (ZM Coulter Counter; Kontron Instruments, Milan, Italy). Aliquots of LND-treated and control cells were differentially processed according to the analyses to be performed.

Flow cytometry. LND-induced apoptosis was detected by flow cytometric analysis of permeabilized, propidium iodide (PI)–stained cells according to the method of Telford et al. (31). Samples (10^6 cells) were washed once in PBS and the pellets resuspended in 80% ethanol at 4°C for at least 60 min. For detection of apoptosis, fixed cells were centrifuged, resuspended in PBS (4×10^6 cell/ml), and maintained at 37°C for 20 min before staining with a 2× solution of 0.1% Triton X-100, 0.1 mM EDTA$(Na)_2$, 50 μg/ml RNase A, and 50 μg/ml PI in PBS. Samples were stored in the dark at room temperature and analyzed with a FACScan® (Becton Dickinson & Co., San Jose, CA). For cell cycle analysis, 2×10^5 fixed cells were centrifuged, resuspended in a solution containing 750 μg/ml RNase A, and 50 μg/ml of PI. At least 2×10^4 events/sample were acquired in list mode by a Consort 32 minicomputer (Hewlett-Packard Co., Palo Alto, CA). Cell percentages in the different phases of the cell cycle were estimated according to Fox's method (32).

Morphological examination. Cell morphology was evaluated by light microscopy. Cytocentrifuge preparations were stained with Mayer's 0.1% ematoxylin-eosin and coverslipped. Apoptotic cells were identified using previously defined criteria (33, 34).

DNA fragmentation analysis. DNA fragmentation in cells undergoing apoptosis was assayed according to Skladanowski and Konopa (35) with minor modifications. Briefly, 5×10^6 cells were lysed in 1 ml lysing buffer containing 10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 10 mM NaCl, 0.5% SDS, and 0.5 μg/ml proteinase K for 1 h at 50°C. High molecular weight DNA was precipitated by addition of 1 M NaCl overnight at 4°C. Samples were centrifuged for 30 min at 2,700 *g*. Supernatants were collected, 2.5 vol 95% ethanol was added and DNA was precipitated overnight at −20°C. DNA pellets were resuspended in 20 μl of 10 mM Tris-HCl, pH 7, 15 mM NaCl, 1 mM EDTA, and 5 μl of 1 mg/ml RNase A. DNA was electrophoresed in 2% agarose gel containing ethidium bromide (1 μg/ml) and photo-

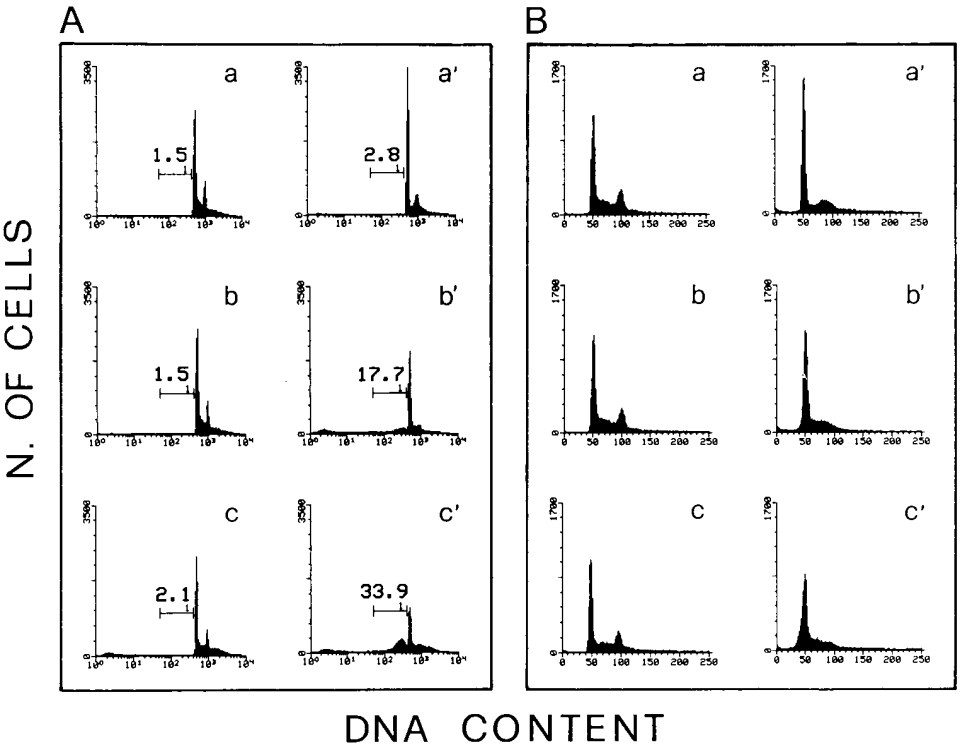

Figure 1. Flow cytometric analysis of MCF-7 ADR^R cells in the absence (*a*, *b*, *c*) and presence of 50 μg/ml LND for 12 (*a'*), 24 (*b'*), and 48 h (*c'*). (*A*) Logarithmic representation of fluorescence intensity obtained by PI-staining to distinguish live from apoptotic cells. Sub-G1 peaks are clearly evident after 24 (*b'*) and 48 h (*c'*) of LND treatment. The numbers inside the graphics indicate the percentages of apoptotic cells. (*B*) The linear PI-fluorescence of cell cycle distribution after LND treatment for 12 (*a'*), 24 (*b'*), and 48 h (*c'*). An increase of G0/G1 phase paralleled by a decrease of S+G2/M phases is evident after 12 h of LND treatment.

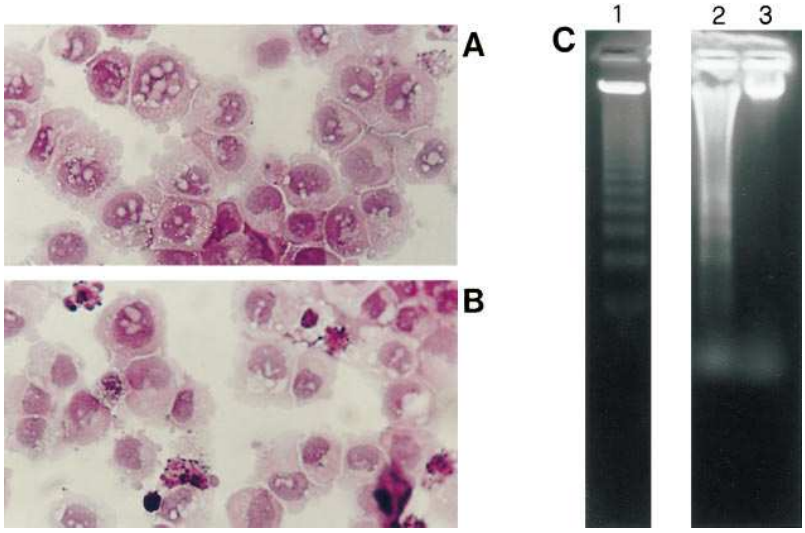

Figure 2. Effect of LND (50 μg/ml for 30 h) on MCF-7 ADR^R morphology and internucleosomal DNA fragmentation. Mayer's 0.1% ematoxylin-eosin–stained preparations of untreated (*A*) and LND-treated (*B*) cells. Note chromatin condensation and chromatin granules. (*C*) Ethidium bromide–stained agarose gel of electrophoresed DNA from MCF-7 ADR^R cells untreated (lane *3*) or LND treated (lane *2*). Lane *1* contains camptothecin-treated HL60 cells (0.15 μM for 6 h) as positive control. LND-treated MCF-7 ADR^R cells present the characteristic pattern of DNA fragmentation.

graphed under ultraviolet illumination. HL60 cells treated for 6 h with 0.15 μM camptothecin were used as positive control (13).

Western blotting. For preparation of total cell lysates, 10^6 cells were incubated at 4°C for 30 min in lysis buffer with ionic detergent (2% SDS, 20 mM Tris, pH 8.0, 2 mM PMSF), sonicated, and assayed for protein concentration using the bicinchoninic acid protein assay reagent (BCA; Pierce Chemical Co., Rockford, IL). For each sample, equal amounts of total proteins were loaded.

For differential preparation of proteins from nuclei and cytoplasm, 10^6 cells were first lysed at 4°C for 30 min in lysis buffer without ionic detergent (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 10 mM EDTA, 0.5% Nonidet P-40) and centrifuged at 500 *g* for 30 min. Supernatants were collected and nuclei were lysed at 4°C for 30 min in lysis buffer with ionic detergent and sonicated. Both cytoplasmic and nuclear proteins were loaded.

Proteins obtained from total cells, cytoplasms, or nuclei were separated on SDS-PAGE: 4–15% gradient for p53 and bcl-2, 17% for p21$^{waf1/cip1}$, and transferred to nitrocellulose (Immobilon TM PVDF membrane; Millipore Corp., Bedford, MA) at 30 V overnight. Anti–p53 mAb (clone PAb 1801; Oncogene Science Inc., Manhasset, NY), anti–bcl-2 mAb (clone 124; Dako SA, Glostrup, Denmark), and anti–p21$^{waf1/cip1}$ mAb (clone EA10; Oncogene Research Products, Cambridge,

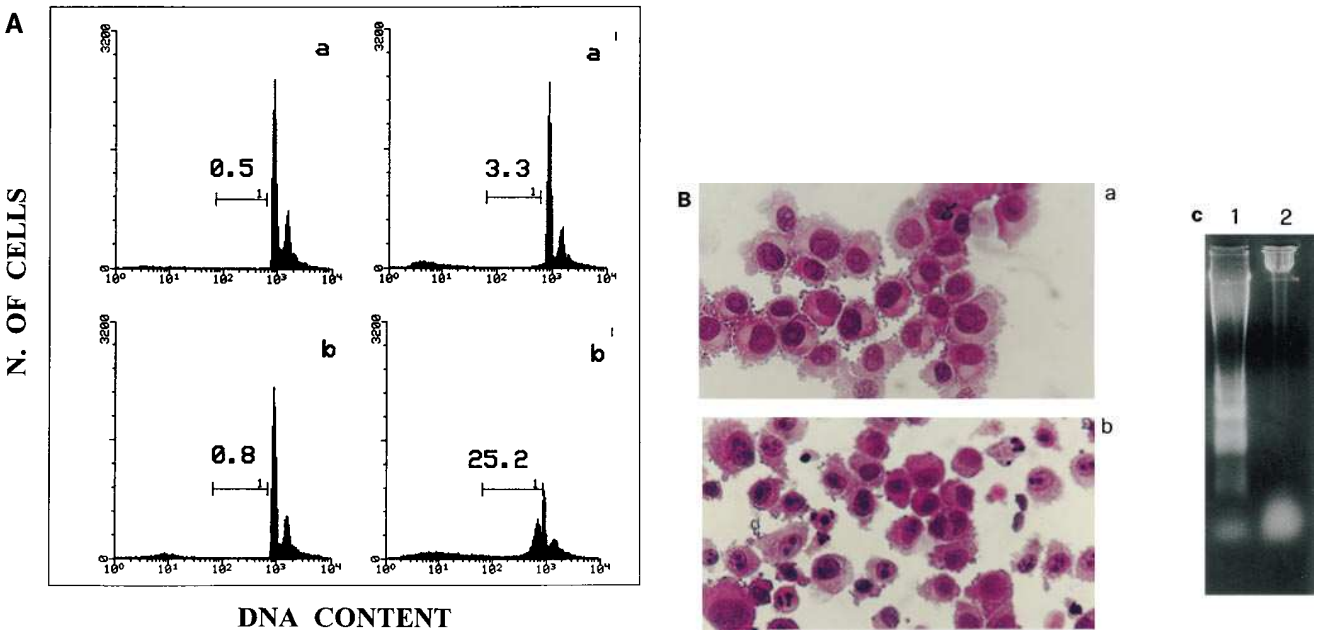

Figure 3. Effect of LND on DNA content distribution (*A*), morphology (*B*), and internucleosomal DNA fragmentation (*C*) of LB9 cells. (*A*) Flow cytometric analysis of LB9 cells in the absence (*a* and *b*) and the presence of 50 μg/ml LND for 24 (*a'*), and 48 h (*b'*). Logarithmic representation of fluorescence intensity was obtained by PI staining to distinguish live from apoptotic cells. Sub-G1 peaks are clearly evident after 48 h (*b'*) of LND treatment. The numbers inside the graphics indicate the percentages of apoptotic cells. (*B*) Mayer's 0.1% ematoxylin-eosin–stained preparations of untreated (*a*) and LND-treated cells (*b*). Note chromatin condensation and chromatin granules. (*C*) Ethidium bromide–stained agarose gel of electrophoresed DNA from LB9 cells, untreated (lane *2*) or LND treated (lane *1*). LND-treated LB9 cells present the characteristic pattern of DNA fragmentation.

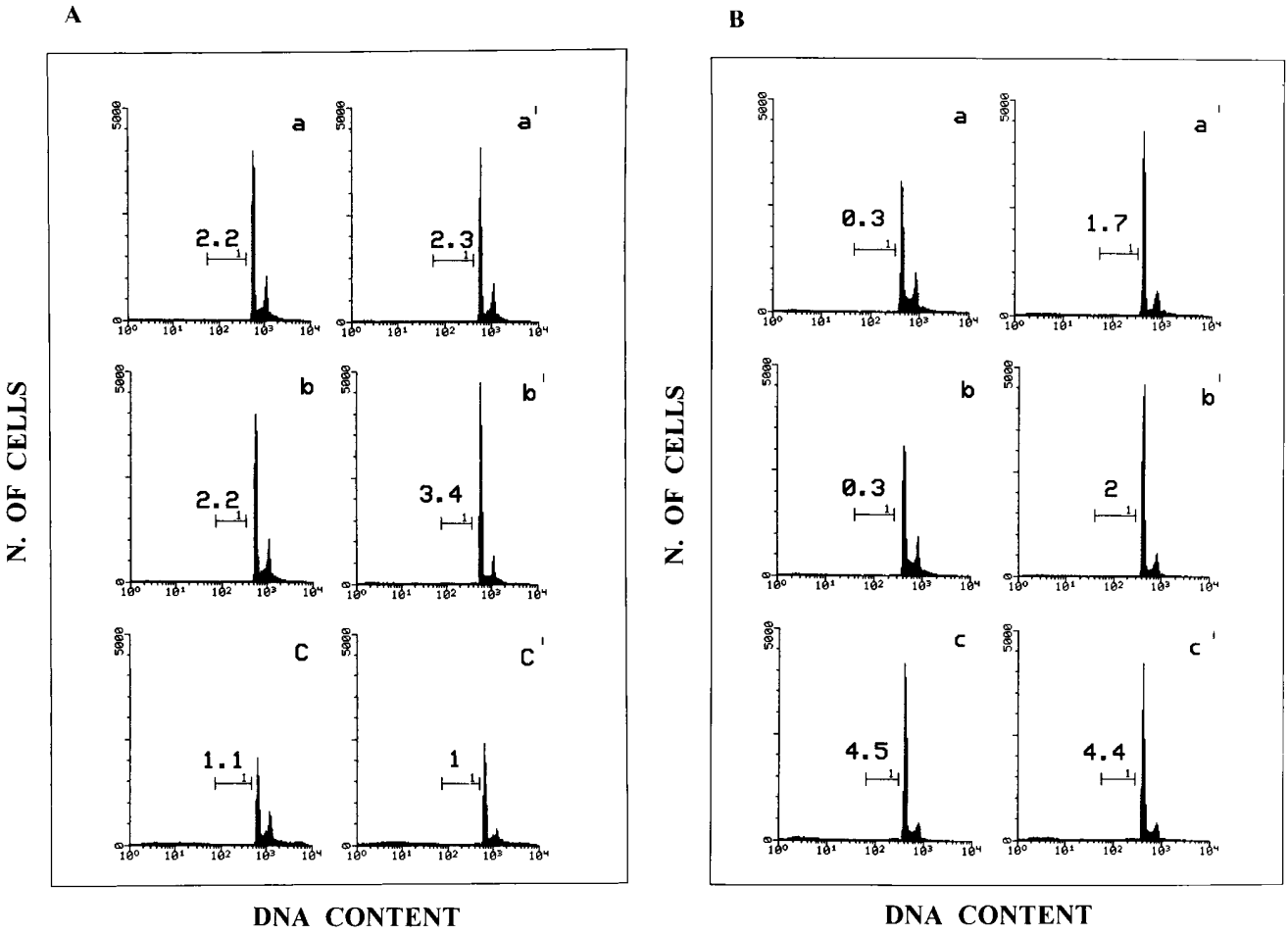

Figure 4. Flow cytometric analysis of MCF-7 WT (*A*) and LI (*B*) cells untreated (*a*, *b*, and *c*) or treated with 50 μg/ml LND for 24 (*a'*), 48 (*b'*), and 72 h (*c'*). The PI-fluorescence is reported on a logarithmic scale. The absence of any sub-G1 peak upon LND treatment is evident.

MA) were used at 1:500, 1:200, and 1:100 dilutions, respectively. Horseradish-peroxidase–conjugated anti–mouse antibody (172-1011; Bio-Rad Laboratories, Hercules, CA) was used according to manufacturer's instructions. Detection was accomplished using the enhanced chemiluminescence detection kit (Amersham Corp., Arlington Heights, IL). To check the amount of proteins transferred to nitrocellulose membrane, β-actin was used as control and detected by an anti–human β-actin mAb (clone JLA2O; Oncogene Science). The relative amounts of the transferred p53, $p21^{waf1/cip1}$, and bcl-2 proteins were quantified by scanning the autoradiographic films with a gel densitometer scanner (Bio-Rad Laboratories) and normalized to the related β-actin amounts.

Immunoprecipitation. Approximately 2×10^6 cells were washed three times in cold PBS and incubated for 30 min on ice in lysis buffer (150 mM NaCl, 50 mM Tris, pH 8, 5 mM EDTA, 1% Nonidet P-40, 1 mM PMSF). The lysates were centrifuged at 14,000 rpm for 5 min and the pellets discarded. Lysates were subjected to immunoprecipitation by incubation for 60 min at 4°C with the anti–p53 PAb 1620, which recognizes only the wt-p53 protein (Ab-5; Oncogene Science Inc., Uniondale, NY). Immunocomplexes were precipitated with Immunopure Plus protein A (Pierce Europe B.V., Oud Beijerland, The Netherlands), washed four times with lysis buffer, boiled for 5 min in sample buffer, and analyzed on 4–15% SDS-PAGE. Western blot analyses of immunocomplexes were performed as described above.

Transfection. MCF-7 ADR^R cells ($\sim 2 \times 10^6$ cells/250 μl medium) were transfected by electroporation (0.25 V, 960 μF) with a Gene Pulser (Bio-Rad Laboratories), with the expression vector pN53cG (Val135) carrying the neoresistance gene and the *p53* Val^{135} mutant gene (36). The latter gene encodes a temperature-sensitive p53 (ts-p53) protein that has a mutant configuration at 37°C and wild-type activity at 32°C (36, 37). As control, MCF-7 ADR^R cells were transfected with the plasmid pRSVneo carrying the neoresistance gene alone. Transfected clones were picked up after 20 d of selection in G418-containing medium (800 μg/ml) and p53 expression was tested by indirect immunofluorescence. One neotransfected clone (MAN-8) and three clones expressing high levels of exogenous ts-p53 protein (MA53-31, MA53-32, and MA53-36) were selected for the experiments.

Indirect immunofluorescence. Approximately 5×10^4 cells were plated in 35-mm tissue culture dishes and incubated at 37°C. At 80% confluence, the cells were fixed with absolute methanol at −20°C for 30 min, rehydrated in PBS, and preblocked for 30 min at room temperature in PBS containing 3% FCS. The cells were incubated for 1 h at 37°C with mAb PAb 122 (Boehringer-Mannheim Italia S.p.a., Milano, Italy), which recognizes a conserved denaturation-stable epitope of the p53 protein. After washing, cells were incubated for 45 min with goat anti–mouse, affinity purified, FITC-conjugated IgG $(Fab)_2$ (Cappel Laboratories, West Chester, PA), and washed with PBS. Positive cells were detected by fluorescence microscope.

Results

LND treatment induces apoptosis in drug-resistant cells. Since the drastic reversion of the ADR-resistant phenotype af-

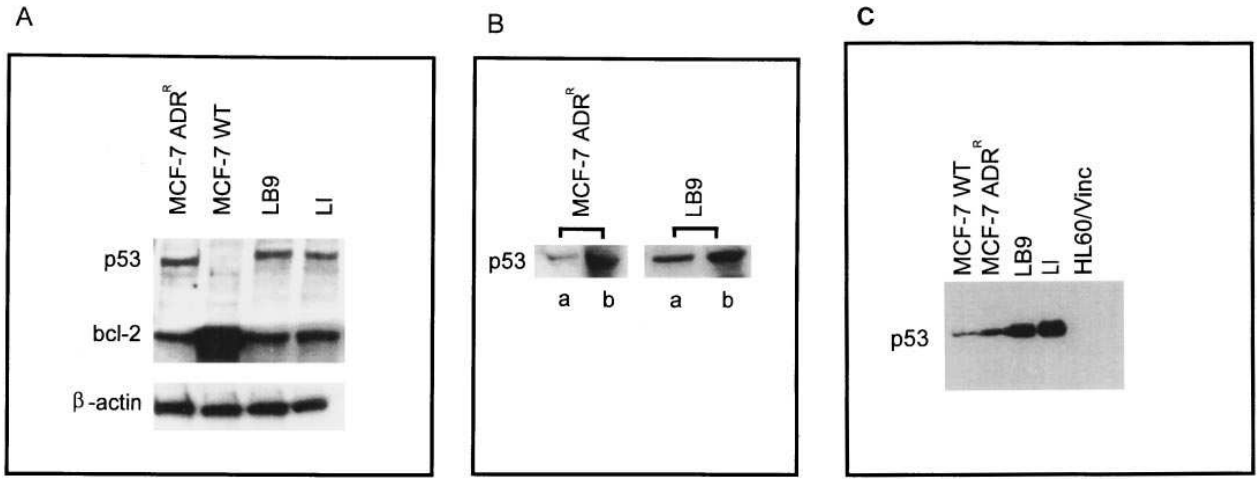

Figure 5. Western blot analysis of p53 and bcl-2 proteins in MCF-7 ADR^R, MCF-7 WT, LB9, and LI cells (*A*), p53 subcellular localization in MCF-7 ADR^R and LB9 cells (*B*), Western blot analysis of immunoprecipitated wild-type p53 protein from MCF-7 WT, MCF-7 ADR^R, LB9, LI, and HL60/Vinc cells (*C*). (*A*) Cell lysates from the indicated cells, containing equal amounts of proteins, were loaded on denaturing 4–15% SDS-PAGE. The relative amounts of the transferred p53 and bcl-2 proteins were quantified and normalized to the correspondent β-actin amounts. (*B*) The p53 subcellular localization was evaluated on proteins from nuclei (*a*) and cytoplasm (*b*), obtained from 10^6 MCF-7 ADR^R and LB9 cells, and separated on denaturing 4–15% SDS-PAGE. (*C*) Wild-type p53 protein was precipitated from cells lysates with an anti–p53 monoclonal antibody that recognizes an epitope only present in the wt-p53 protein and decorated with an anti–p53 monoclonal antibody. HL60/Vinc cells are used as negative control.

ter LND and ADR combination treatment (4) cannot be completely explained by the increased intracytoplasmic levels of ADR (10), we evaluated whether LND alone might induce cytopathic effects in ADR-resistant cells. Exponentially growing MCF-7 ADR^R cells were treated for 12, 24, and 48 h with 50 μg/ml of LND, the dose able to revert ADR resistance (4). A pool of detached and adherent cells was analyzed for DNA content by cytofluorimetry upon PI staining (Fig. 1 *A*). After 24- and 48-h exposures to LND, sub-G1 peaks were evident, the dead cells being about 18 and 34% of total populations, respectively. Since DNA content below that of G0/G1 suggests cell death by apoptosis (31, 38), we performed a series of experiments to better define LND-induced effect.

Induction of apoptosis is frequently associated with cell cycle modifications (39). Analysis of cell cycle phase distributions of MCF-7 ADR^R cells treated with LND for the same periods indicated above showed cell accumulation in the G0/G1 compartment (about 67% of the treated population compared to 37% of the untreated one) as early as 12 h after LND exposure (Fig. 1 *B*). The G0/G1 arrest is accompanied by depletion of both the S and G2/M phases of the cell cycle.

To confirm the apoptotic nature of the observed cell death, morphological and molecular parameters were evaluated. After 30 h, the LND-treated population (Fig. 2 *B*) shows the characteristic morphological features of apoptosis. Chromatin condensation at the margins of the nuclear membrane, as well as chromatin granules resulting from DNA fragmentation are visible. The pathognomonic feature of apoptosis is the cleavage of DNA in the internucleosomal regions of chromatin. Analysis of DNA fragmentation in MCF-7 ADR^R cells treated with LND for 30 h showed DNA ladder formation (Fig. 2 *C*, lane *2*), which was absent in untreated cells (Fig. 2 *C*, lane *3*).

To assess whether apoptosis occurs in other resistant cell lines, we evaluated LND effects on cells with a different histotype and resistance to other anticancer drugs. A glioblastoma multiform BCNU-resistant cell line (LB9) was treated with LND for different periods of time. The cells were then analyzed for their DNA content, pattern of internucleosomal cleavage of genomic DNA and morphology. The results shown in Fig. 3 *A* indicate that a hypodiploid peak is evident in LB9 48 h after LND treatment (dead cells being ~ 25% of the total population). As observed in MCF-7 ADR^R cells, induction of apoptosis in LB9-resistant cells is accompanied by a G0/G1 accumulation, being the increase of cells in G0/G1 phase of about 45% vs control (data not shown). Morphological features of apoptosis and DNA fragmentation are evident in LB9 cells treated with LND for 48 h (Fig. 3 *B*).

Since LND induced apoptosis in resistant cells, we investigated whether LND could also activate the apoptotic program in parental-sensitive cells. To this purpose, MCF-7 WT and LI parental cell lines were treated with 50 μg/ml of LND for different times and both DNA content and cell cycle distribution were evaluated. Fig. 4 shows that no sub-G1 peak was observed in MCF-7 WT (Fig. 4 *A*) and LI (Fig. 4 *B*) cells in spite of longer time exposure. Furthermore, cell cycle phase distribution is similar between controls and LND-treated cells both in MCF-7 WT and LI parental cells (data not shown).

Bcl-2 and p53 protein expression in drug-resistant and -sensitive cells. It has been demonstrated that *bcl-2* inhibits and *p53* mediates apoptosis (14, 40, 41). In view of the different effects observed after LND treatment in resistant and sensitive cells, we sought to verify the expression of these genes in all four cell lines (Fig. 5 *A*). We found that LI and LB9 cells express similar levels of bcl-2 protein, whereas MCF-7 WT cells express bcl-2 protein at greater levels than those observed in MCF-7 ADR^R cells. The observation that sensitive and drug-resistant glioblastoma cells express similar levels of bcl-2 suggests that the unresponsiveness of sensitive cells to LND-induced apoptosis might not only be due to bcl-2 overexpres-

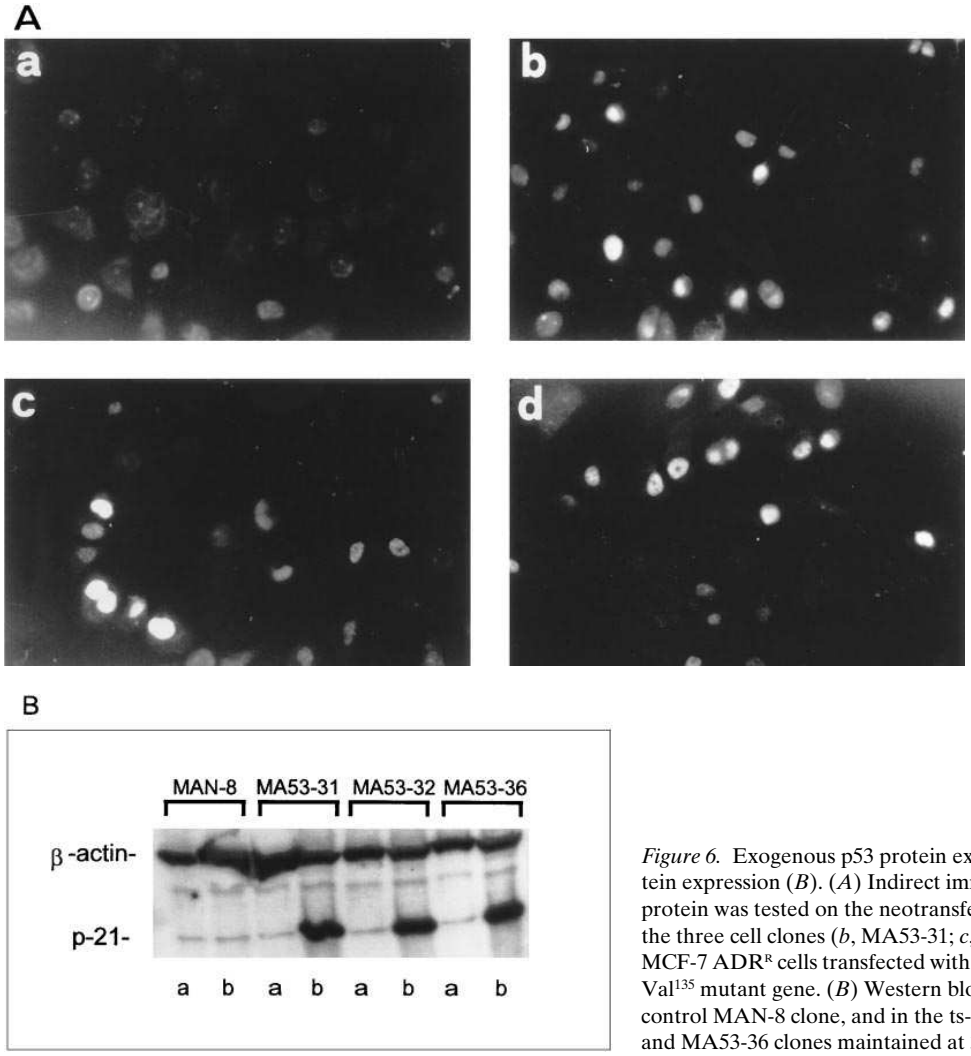

Figure 6. Exogenous p53 protein expression (*A*) and p21$^{waf1/cip1}$ (*p21*) protein expression (*B*). (*A*) Indirect immunofluorescence of exogenous p53 protein was tested on the neotransfected MAN-8 control cells (*a*), and on the three cell clones (*b*, MA53-31; *c*, MA53-32; *d*, MA53-36) derived from MCF-7 ADR^R cells transfected with the expression vector carrying the p53 Val^{135} mutant gene. (*B*) Western blot analysis of p21$^{waf1/cip1}$ gene in the control MAN-8 clone, and in the ts-p53 expressing MA53-31, MA53-32, and MA53-36 clones maintained at 37°C (lanes *a*) and at 32°C (lanes *b*).

sion (21, 22). Moreover, comparable levels of p53 protein were detected in LI and LB9 cells, whereas the levels of its expression were high in MCF-7 ADR^R and not detectable in MCF-7 WT cells in the experimental conditions used. p53 can either mediate or protect cells from apoptosis, depending whether it is wild type or mutant (20, 42). Mutant p53 proteins are generally more stable than wt-p53 and cells with high p53 protein levels generally express mutated, or at least inactivated forms of p53 (43). Given these observations, the increased p53 levels found in MCF-7 ADR^R cells, which are sensitive to the LND-induced apoptosis, were rather surprising. We investigated the subcellular localization of p53 protein in both resistant cell lines. Western blots (Fig. 5 *B*) of cytoplasmic and nuclear proteins from MCF-7 ADR^R and LB9 cells show that p53 is segregated in the cytoplasm and, consequently, probably inactive (44). To ascertain the phenotype of the *p53* gene of sensitive and drug-resistant cell lines, immunoprecipitation experiments were performed. A monoclonal antibody that recognizes wild-type p53 protein was used, and the immunocomplexes were detected by western blot analysis. Fig. 5 *C* demonstrates that all cell lines express p53 in wild-type configuration.

The observation that the cells express a wt-p53 protein independently of their responsiveness to LND-induced apoptosis suggests that p53 protein is not involved in LND-mediated apoptosis.

LND-induced apoptosis is independent of p53 protein. To confirm the p53-independence of LND-induced apoptosis, we evaluated LND effects in drug-resistant cells lacking the endogenous p53 protein (vincristine-resistant derivative HL60 cell line), and in MCF-7 ADR^R cells transfected with an expression vector carrying the p53 Val^{135} mutant gene (36). This gene encodes a temperature-sensitive p53 protein that has a mutant configuration at 37°C. This protein acts as a dominant negative of the wt-p53 protein (36, 37) and partially protects from wt-p53–mediated apoptosis (45, 46). In contrast, the ts-p53 protein acquires a wild-type configuration at 32°C (36, 37) and accelerates p53-mediated apoptosis (43, 44). Three transfected cell clones (Fig. 6 *A*, *b*, MA53-31; *c*, MA53-32; *d*, MA53-36) expressing high levels of exogenous ts-p53 were selected by indirect immunofluorescence analysis, together with a neotransfected clone (Fig. 6 *A*, *a*, MAN-8). To exclude the possibility that the function of wt-p53 overexpressed in MCF-7 ADR^R cells is impaired due to its sequestration in the cytoplasm, we examined whether the transcription levels of the p21$^{waf1/cip1}$ gene was induced in transfected cells at the permissive temperature. It is well documented that overexpression of wt-p53 pro-

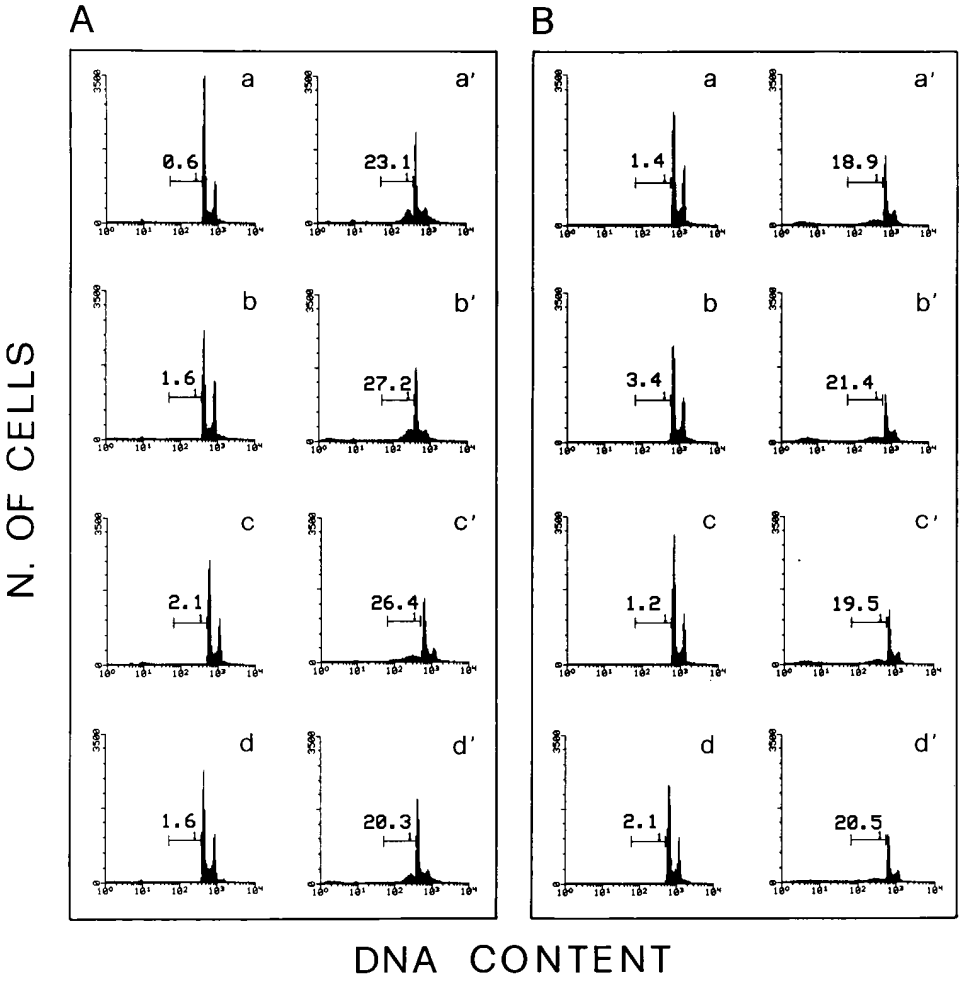

Figure 7. Flow cytometric analysis of ts-p53–tranfectants maintained at both 37°C (*A*) and 32°C (*B*). The cells were incubated for 48 h without (*a–d*) or with 50 μg/ml of LND (*a′–d′*). Neotransfected MAN-8 cells (*a* and *a′*) and the three ts-p53-expressing cell clones (*b* and *b′*, MA53-31; *c* and *c′*, MA53-32; *d* and *d′*, MA53-36) are reported. Logarithmic representation of PI-fluorescence was obtained as described in Fig. 1 *A*. Sub-G1 peaks are clearly evident upon LND treatment in all cell clones. The numbers inside the graphics indicate the percentage of apoptotic cells.

tein increases transcription of the $p21^{waf1/cip1}$ gene (47). Western blot analysis of $p21^{waf1/cip1}$ in the three ts-p53–expressing clones maintained at either 37 or 32°C are reported in Fig. 6 *B*. We consistently found $p21^{waf1/cip1}$ up regulation only in MCF-7 ADR^R derivatives transfected with ts-p53 and cultured at the permissive temperature, indicating that these cells express a p53 that acts as a wild-type protein. The four clones cultured either at 37 or 32°C were treated with LND (50 μg/ml) and the DNA content was evaluated after 48 h (Fig. 7). Neither a reduction of the apoptotic rate at 37°C (Fig. 7 *A*), nor an increased sensitivity to LND at 32°C (Fig. 7 *B*) were detected. Evaluation of cell cycle phase distribution, cell morphology, and DNA fragmentation were similar to those reported for parental MCF-7 ADR^R cells (data not shown).

We also studied the LND effects on the vincristine-resistant human promyelocytic leukemia cell line (HL60/Vinc) lacking the endogenous p53 protein (Fig. 5 *C*). Fig. 8 shows sub-G1 peaks in treated cells, the death cells of ~51% after 48 h of treatment. Together these results clearly demonstrate that LND induces apoptosis independently of the *p53* gene.

Discussion

The inability to activate the apoptotic program is a newly recognized pathway of drug resistance, which results in a significant proportion of treatment failures. We previously demonstrated that LND can play a significant role in overcoming ADR resistance in MCF-7 ADR^R breast cancer cell line (4). In the present study, we show that LND exerts its action by directly inducing apoptosis of these cells. In particular, after LND treatment we found sub-G1 peaks in DNA-content histograms and patterns of internucleosomal cleavage of genomic DNA. Morphological examination confirms cell death by apoptosis. The evidence that LND induces apoptosis might explain why LND in combination with ADR was able to almost completely suppress the survival of MCF-7 ADR^R cells at intracellular concentrations of ADR that reduce the survival of MCF-7 WT cells to only 50% (4). Furthermore, LND-induced apoptosis can also explain why the increased intracellular levels of ADR determined by LND treatment were limited to the cytoplasm (10). Hence, besides ADR, the LND-activated apoptosis pathway could play a role in reducing cell survival of resistant cells.

To assess whether LND induction of apoptosis is a common feature of drug-resistant cells, we also studied LND effects on tumor cells of different histotypes where resistance is not related to the MDR-1 gene. DNA ladder, DNA content, and morphological analysis demonstrated that LND induces apoptosis also in a BCNU-derivative glioblastoma multiform cell line. Therefore, we investigated LND effects in the corresponding sensitive parental cell lines (MCF-7 WT and LI). The data presented here show that drug-sensitive parental cells are resistant to LND-mediated apoptosis.

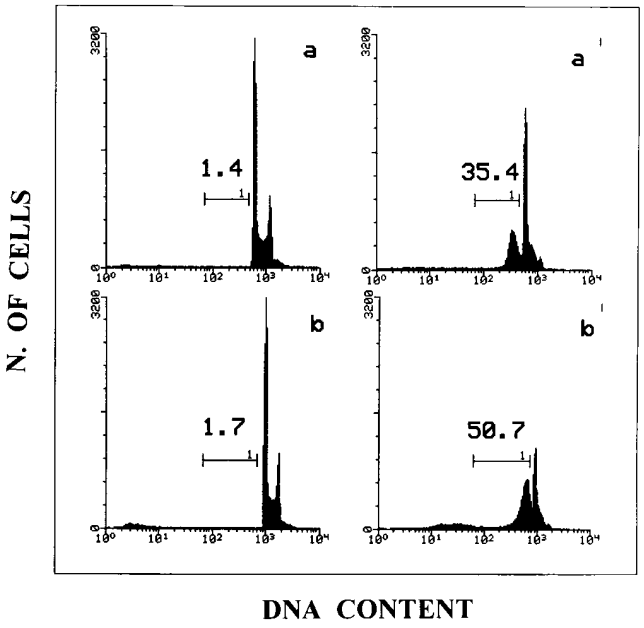

Figure 8. Flow cytometric analysis of HL60/Vinc cells in the absence (*a* and *b*) and presence of 50 μg/ml LND for 24 (*a′*), and 48 h (*b′*). Logarithmic representation of fluorescence intensity was obtained by PI staining to distinguish live from apoptotic cells. Sub-G1 peaks are clearly evident after 24 (*a′*) and 48 h (*b′*) of LND treatment. The numbers inside the graphics indicate the percentages of apoptotic cells.

By studying whether LND-induced apoptosis was associated with cell cycle perturbations, we found that LND treatment induces an early accumulation of cells in the G0/G1 compartment in the two drug-resistant cell lines. These findings, while indicating that drug-sensitive cells are not prone to LND-induced apoptosis, suggest that LND might induce apoptosis through a dissociation of normally integrated cell cycle events.

Even though several chemotherapeutic agents exert their cytotoxic activity by determining a p53-mediated apoptotic cell death (14, 20), we show that LND-induced apoptosis is independent of this protein. The finding that p53 protein is probably inactive in all cells used for our studies, because of its segregation in the cytoplasm (44), supports this observation. Further evidence is the induction of apoptosis by LND in cells lacking endogenous p53 (HL60/Vinc), and the transfection-mediated overexpression of a ts-p53 mutant protein in MCF-7 ADR^R cells. The sensitivity to LND-induced apoptosis is not modified by the dominant-negative effects generated by ts-p53 at 37°C or by the wt-p53 functions at 32°C. Together, these data indicate that, despite the presence of active or inactive p53 protein, drug-resistant cells respond to LND by undergoing apoptosis. This p53 independence might be explained by the fact that LND probably acts at the cytoplasmic level, while p53 is usually reported to mediate apoptotic cell death after DNA damage (48).

It is generally believed that anticancer agents may simply activate an apoptotic program intrinsic to sensitive cells. To ascertain whether LND is a general apoptotic inducer or whether it activates an inherent apoptotic program, we characterized drug-sensitive and -resistant cell lines for the expression of genes involved in apoptotic pathways. The protooncogene *bcl-2* can protect various types of cells from both normal and experimentally induced apoptosis (21, 22). No apparent correlation was found between bcl-2 expression and cell response to LND-induced apoptosis. Indeed, equivalent amounts of bcl-2 protein were observed in LI and LB9 cells, but more bcl-2 protein was detected in MCF-7–sensitive than in –resistant cells (MCF-7 ADR^R). This observation suggests that the level of bcl-2 expression does not influence the outcome of LND effect in sensitive and drug-resistant cells. However, other members of the bcl-2 family need to be evaluated to ascertain bcl-2 pathway independence of LND-induced apoptosis.

Apoptosis is characterized by a loss of mitochondrial function (39, 49, 50). This might mean mitochondria has an important function in regulating apoptosis. However, data to support this hypothesis are not yet available. Interestingly, it has been reported by Floridi and Lehninger (8) that the main targets of LND are mitochondria and that LND impairs mitochondrial functions. Furthermore, it has been suggested that bcl-2 might protect cells from apoptosis by altering mitochondrial functions (39, 49, 50). However, further studies are required to assess the effective action of LND and bcl-2 on mitochondrial function.

The ability of LND to induce apoptosis might also be due to its capacity to interfere with the cell energy metabolism. Even though several cytotoxic agents induce NAD depletion without affecting ATP (51), some compounds might induce death as a result of depletion of NAD followed by depletion of ATP (11, 52). Furthermore, it was previously shown that MCF-7 ADR^R cells exhibit an enhanced rate of glycolysis, and an increased demand for ATP production, compared to MCF-7 WT cells (53). Moreover, LND decreases glucose use, aerobic glycolysis, and ATP content in both cell types being the effect significantly higher on resistant cells (54). Hence, we can hypothesize that the divergent susceptibility to LND-induced apoptosis of resistant and sensitive cells might be due to the different LND effects on energetic metabolism.

In conclusion, our data demonstrate for the first time that LND is able to induce p53-independent apoptosis in three drug-resistant tumor cells. Though the molecular mechanism(s) of LND action need(s) further investigation, our data suggest LND as a good candidate for treatment of tumors carrying p53 alterations and, therefore, apparently resistant to more conventional chemotherapeutic agents (14, 25, 26, 55).

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