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Effects of Liposome-Entrapped Annamycin in Human Breast Cancer Cells: Interference With Cell Cycle Progression and Induction of Apoptosis

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Abstract The effects of liposome-encapsulated annamycin (L-Ann) were investigated in two human breast cancer cell lines, MCF7 and MDA-MB-435. For comparative purposes, doxorubicin (Dx) was used throughout the study. A 4-hour treatment with L-Ann was significantly more active in MDA-MB-435 than in MCF7 cells (IC_{50} values of 0.03 and 0.08 μg/ml, respectively), whereas Dx was equally active in the two cell lines (IC_{50} 0.12 μg/ml). L-Ann induced an accumulation of cells in G_2M phases which was dose-dependent in MDA-MB-435 but not in MCF7 cells. Dx also caused a dose-dependent increase of G_2M cell fraction in MDA-MB-435 cells, whereas a G_2M cell accumulation was observed only after treatment with the highest Dx concentration in MCF7 cells. G_2M phase cell accumulations induced in MCF7 cells by L-Ann or Dx were accompanied by a decrease in cdc2 kinase activity and in cyclin B1 and cdc2 expression. Conversely, in MDA-MB-435 cells exposed to L-Ann or Dx, cdc2 kinase activity, cyclin B1 and cdc2 expression increased in parallel to the increase in the number of cells accumulated in the G_2M phase. L-Ann and Dx induced apoptosis in MDA-MB-435 but not in MCF7 cells. In MDA-MB-435 cells exposed to L-Ann or Dx, no change was observed in the expression of bax, but there was a p53-independent increase in $p21^{waf1}$ expression. In MCF7 cells, treatment with L-Ann or Dx induced an increase in p53 expression with a consequent transactivation of $p21^{waf1}$ and bax. Our results indicate that L-Ann is more cytotoxic than Dx in breast cancer cells and is able to induce apoptosis through p53-independent mechanisms. J. Cell. Biochem. 81:9–22, 2001.

Key words: anthracyclines; cell proliferation; flow cytometry; cdc2 kinase; p53

Annamycin (Ann) is a lipophilic anthracycline antibiotic selected from a series of Dx analogues in an attempt to overcome the dose-limiting cardiotoxicity and resistance that have developed after repeated treatment of tumors with Dx. Ann was designed with four well-defined structural changes compared with Dx [Perez-Soler and Priebe, 1990]: removal of the amino group at position 3′ in the sugar portion and replacement by a hydroxyl group, demethoxylation at position 4′ of the aglycone portion, the presence of iodine at position 2′, and inversion of hydroxyl group configuration at position 4′. The substitutions conferred to Ann a partial lack of cross-resistance with other anthracyclines without modifying the interaction with topoisomerase II. Moreover, they conferred reduced cardiotoxicity to the compound and increased its lipophilicity and affinity for the lipid membrane. Most importantly, since a protonated amino group in the sugar portion is a common feature of chemotherapeutic agents that show cross-resistance in P-glycoprotein (P-gp) positive cells, the substitution of the amino group with a hydroxyl group made Ann a low-affinity substrate for proteins involved in multidrug resistance such as P-gp and the multidrug resistance related protein (MRP). In addition, the high lipophilicity of the compound might cause a preferential association of the drug with lipid cellular components, resulting in a decreased interaction with P-gp and MRP. In fact, several in vitro studies have demonstrated the ability of Ann to circumvent P-gp [Ling et al., 1993a, 1995; Consoli et al., 1996]

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and MRP-mediated resistance to Dx in different tumor models [Perez-Soler et al., 1997].

Since Ann has a high affinity for the lipid membrane and very low solubility in water, different liposomal formulations (unilamellar, multilamellar, and long-circulating liposomes) of the drug have been developed [Priebe and Perez-Soler, 1993; Zou et al., 1995a]. Preclinical studies carried out in animal models [Zou et al., 1995b] showed that liposome carriers reduced the general toxicity of Ann in mice by increasing the LD_{50} by two-fold and reducing vescicant activity and cardiotoxic potential compared with free Ann. Zou et al. [1994] investigated the effect of Ann entrapped in liposomes in different experimental tumor models both in vitro and in vivo and demonstrated that liposomal Ann was more active than Dx by the i.v. route against several tumors models, that the MDR phenotype could be partially overcome by liposomal Ann both in vivo and in vitro, and that the activity of Ann was enhanced by using liposomes, particularly small ones, as delivery systems. Perez-Soler et al. [1994] performed a comparison of the efficacy of free Ann, liposomal Ann, and Dx in KB and KB-V1 cells and found that the effect of L-Ann in vitro was only slightly affected by liposomes, thereby suggesting that the advantage of using liposome-entrapped Ann in vivo was probably due to changes in pharmacokinetics and organ distribution of the drug secondary to liposome entrapment.

Little is known about the effect of Ann on cell cycle and its ability to induce apoptosis in cell lines derived from solid tumors. The only study on apoptosis [Ling et al., 1993b] reported that Ann was able to induce an internucleosomal DNA degradation compatible with programmed cell death in P388 murine leukemia cells.

Our study was undertaken to investigate the effects of a liposome-entrapped Ann formulation currently used in a Phase II clinical trial in breast cancer, in terms of impairment of cell cycle progression, induction of programmed cell death, and interference with proteins involved in these cellular processes, in two human breast cancer cell lines.

METHODS

Chemicals

Dx, obtained from Pharmacia-Upjohn (Uppsala, Sweden), was dissolved in normal saline solution. Free Ann (F-Ann) and liposomal Ann (L-Ann), kindly supplied by Dr. Cossum (Aronex Pharmaceuticals, The Woodlands, Texas), were prepared and synthesized as previously described [Zou et al., 1996]. F-Ann was suspended in 10% dimethyl sulfoxide and 90% normal saline (1 mg/ml stock solution). For the preparation of L-Ann supension, on the day of use, the lyophilized preliposomal powder containing Ann was reconstituted by adding saline to the vial and hand shaking until all lyophilized material was suspensed. Further dilutions were prepared in normal saline.

Cell Lines and Cell Proliferation Studies

The MCF7 human breast cancer cell line was cultured in RPMI 1640 (BioWhittaker, Walkersville, MD) medium-supplemented with 10% heat-inactivated fetal bovine (BioWhittaker) serum and 2 mM glutamine (BioWhittaker). The MDA-MB-435 human breast cancer cell line was cultured in DMEM-F12 (BioWhittaker) medium-supplemented with 5% heat-inactivated fetal bovine serum. MCF7 is characterized by a wild-type p53 gene, and MDA-MB-435 carries a mutated p53 gene (266 GGA→GAA) [O'Connor et al., 1997].

After harvesting in the logarithmic growth phase, cells were seeded in 6-well plates and treated with varying doses of F-Ann, L-Ann and Dx for 4 h. At the end of the treatment, adherent cells were washed with phosphate-buffered saline (PBS, BioWhittaker) and incubated at 37°C in a 5% CO_2-humidified atmosphere for 3 days. Cells were then trypsinized and counted in a particle counter (Coulter Counter, Coulter Electronics, Luton, UK). The percentages of adherent viable cells were determined by the Trypan blue dye exclusion test. Viability always exceeded 95%. Each experimental sample was run in triplicate. The results were expressed as the total number of adherent cells in treated samples compared with control samples in which only normal saline was added. In vitro activities of different drugs were expressed in terms of concentrations able to inhibit cell proliferation by 50% (IC_{50}) and 80% (IC_{80}).

Flow Cytometric Analysis

At different intervals after drug treatment cells were harvested, floating and adherent cells were collected separately, and samples of 1×10^6 of cells were fixed in 70% ethanol.

Before analysis, cells were washed in PBS and stained with the solution A containing 50 μg/ml propidium iodide, 50 mg/ml RNAse, and 0.05% NP40 for 30 min at 4°C and then analyzed with a FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA). The cell cycle distribution was evaluated on adherent cells on DNA plots by CellFit software according to the SOBR model (Becton Dickinson). The analysis of the presence of pre-G_1 apoptotic population was performed on both adherent and floating cell samples.

Cell Lysis and Immunoblotting

Cells (adherent plus floating) were lysed in 1% Nonidet P-40, prepared in PBS containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM 4-(2-aminoethyl) benzensulfonylfluoride (AEBSF), 1 mM Na_3VO_4, 1 mM $NaPPO_4$, and 10 mM NaF. Cell lysates were clarified (30 min, 15,000 rpm), and the resultant supernatants were used for protein analysis. Total cellular lysate (100 μg) was dissolved in 2× sample loading buffer (2% lauryl sulfate sodium salt (SDS), 5% 2-mercaptoethanol, 20% glycerol, 60 mM Tris, pH 6.8, and 0.0025% bromophenol), separated on 12% SDS-polyacrylamide gel and transferred to nitrocellulose. Filters were blocked in PBS with 5% skim milk and then incubated overnight with the primary antibody anti-p53, anti-Bax, anti-Bcl-2, anti-cyclin B_1, and anti-cdc2 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-$p21^{waf1}$ (Oncogene Science, Cambridge, MA), and antiphospho-specific cdc2 (Tyr15) (New England Biolabs, Beverly, MA). Filters were then incubated with the secondary antimouse or antirabbit Ig horseradish peroxidase-linked whole antibody (Amersham Pharmacia Biotech, Uppsala, Sweden). Bound antibody was detected using the enhanced chemoluminescence Western blotting detection system (Amersham). An anti-PCNA monoclonal antibody (Santa Cruz Biotechnology) was used on each blot to ensure equal loading of protein on the gel.

Immunoprecipitation and Assay of Cyclin B_1-Associated cdc2 Kinase Activity

Cells (1×10^6) were washed once with ice-cold PBS and lysed on ice in lysis buffer for kinase assay (1% v/v Nonidet P-40 prepared in PBS containing 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM AEBSF, and 1% bovin serum albumin (BSA)). Cell lysates were clarified (30 min, 15,000 rpm) and 0.5 ml of lysate from 1×10^6 cells was mixed with a mouse monoclonal anti-cyclin B_1 (Santa Cruz Biotechnology) in the presence of 100 μl of a 20% (v/v) protein A-Sepharose slurry (Amersham) followed by rotation for 4 h at 4°C. The immune complexes were then washed twice with lysis buffer for kinase assay and then twice in the same buffer minus BSA. The cyclin B1 immunoprecipitates were incubated with 3 μg of histone H1 (Boehringer Mannheim, Mannheim, Germany) in 20 μl of kinase buffer containing 20 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 5 μM cold ATP, and 10 μCi of [γ^{32}P]ATP for 20 min at 30°C. The reaction was terminated by adding an equal volume of 2× SDS sample loading buffer. The mixture was then boiled for 5 min before loading onto a 12% SDS-polyacrylamide gel. Following autoradiography, reactions were quantified by densitometry. Kinase activities of treated samples were expressed as percentage variation with respect to kinase activities of individual controls.

Evaluation of Apoptotic Morphology by Fluorescence Microscopy

At different intervals after drug treatment, cells were harvested; floating and adherent cells were collected separately, washed in PBS and stained with the solution A. After staining, the slides were observed under fluorescence microscopy. The percentage of apoptotic cells was determined by scoring at least 500 cells on each sample [Orlandi et al., 1999].

DNA Agarose Gel Electrophoresis

Adherent and floating cells (3×10^6) were lysed in a solution containing 10 mM EDTA, 5 mM Tris–HCl (pH 8.0) and 0.5% Triton X-100 for 30 min on ice. Samples were centrifuged and the supernatant (low molecular weight DNA) was separated from the pellet (high molecular weight DNA). Both fractions were digested with RNAse A (500 U/ml) for 1 h at 37°C and 1% w/v SDS detergent containing 0.5 mg/ml proteinase K for 3 h at 50°C. The samples were extracted once with phenol and once with phenol chloroform isoamyl alcohol (25:24:1, v:v:v), precipitated with 2.5 vol of ethanol and 0.1 vol of sodium acetate (3 M, pH 5.2), and then dissolved in TE buffer [10 mM Tris–HCl (pH 7.5), 1 mM EDTA (pH 8.0)]. DNA was electrophoresed in 1.5% agarose gel in 1× TBE buffer (89 mM Tris base, 89 mM

boric acid, 2 mM EDTA). The gel was stained with 10 μg/ml ethidium bromide for 15 min, destained for 20 min in water, and visualized under UV light.

RESULTS

Effects of Ann and Dx on Cell Proliferation and Cell Cycle Progression

Results from the cytotoxicity study (Fig. 1; Table I) showed that a 4-h treatment with L-Ann was slightly more active than F-Ann in both cell lines. Moreover, L-Ann was almost three times more cytotoxic ($P < 0.01$) in MDA-MB-435 than in MCF7 cells (Table I). Dx showed a superimposable activity in both cell lines and was less active than L-Ann. Such a difference was significant ($P < 0.01$) in MDA-MB-435 but not in MCF7 cells (Table I). For further experiments, only the liposome-encapsulated form of L-Ann was used for comparison with Dx. Specifically, two concentrations of each drug close to IC_{50} and IC_{80} values were chosen: in MDA-MB-435, 0.03 μg/ml and 0.1 μg/ml for L-Ann and 0.12 and 0.48 μg/ml for Dx; in MCF7, 0.1 and 1.5 μg/ml for L-Ann and 0.12 and 0.36 μg/ml for Dx.

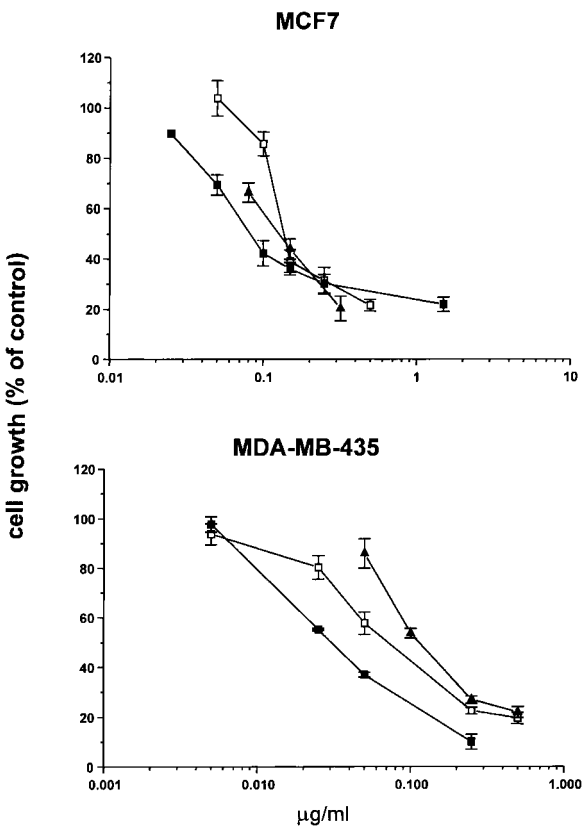

Fig. 1. Dose-response curves of MCF7 and MDA-MB-435 cells treated with F-Ann (□), L-Ann (■) or Dx (▲). Cells were exposed to each drug for 4 h, washed and incubated in drug-free medium for an additional 72 h. The effects of drugs were determined as variation in the number of treated cells compared with control cells. Values represent the mean (±SD) of at least three independent experiments.

TABLE I. Sensitivity of Breast Cancer Cells to Drugs[a]

Drug	MCF7 IC_{50} (μg/ml)	MDA-MB-435 IC_{50} (μg/ml)
F-Annamycin	0.14 ± 0.05	0.07 ± 0.03
L-Annamycin	0.08 ± 0.01	0.03 ± 0.00[b]
Doxorubicin	0.12 ± 0.053	0.12 ± 0.007[c]

[a]Data were extrapolated from the growth-inhibition curves obtained with the two drugs and represent mean values ±SD from at least three independent experiments.
[b]Significantly different from L-Annamycin in MCF7 ($P < 0.01$, Student's t-test).
[c]Significantly different from L-Annamycin in MDA-MB-435 ($P < 0.01$, Student's t-test).

DNA flow cytometry analysis was performed to determine whether cell cycle perturbation could be responsible for the different cytotoxic activity induced by the two drugs. The kinetics of cell cycle distribution of cells treated with L-Ann and Dx are shown in Table II. A 4-hour treatment of MCF7 cells with the IC_{50} concentration of L-Ann induced an accumulation of cells in G_2M which was maximum after 24 h from the beginning of the treatment and partially resolved after 72 h. Conversely, after treatment with the IC_{80} concentration of L-Ann, a small but stable increase in the G_2M phase cell fraction was detected at all times considered. To investigate whether the highest L-Ann concentration (IC_{80}) could induce G_2M blocks at intervals earlier than 24 h, we assessed cell cycle perturbations after 4 and 8 h from the beginning of L-Ann treatment. However, only a slight increase in S-phase cell number was appreciable, without any accumulation of cells in the G_2M compartment (data not shown). After treatment with the IC_{50} Dx concentration, MCF7 cells were stably blocked in $G_{0/1}$ until 72 h. In contrast, treatment with the highest Dx concentration (IC_{80}) induced G_2M phase cell accumulations superimposable to those induced with the IC_{50} L-Ann concentration in the cell line.

As regards MDA-MB-435, a dose-dependent accumulation of cells in the G_2M phase was observed after exposure to L-Ann. Specifically,

TABLE II. Cell Cycle Perturbation Induced by L-Ann and Dx[a]

	Time								
	24 h			48 h			72 h		
	$G_{0/1}$	S	G_2M	$G_{0/1}$	S	G_2M	$G_{0/1}$	S	G_2M
MCF7									
Control	44 ± 3	45 ± 2	11 ± 2	48 ± 5	40 ± 3	12 ± 2	47 ± 3	40 ± 2	13 ± 2
L-Ann, 0.1 μg/ml	35 ± 2	6 ± 2	59 ± 3	42 ± 0.5	22 ± 4	37 ± 4	45 ± 2	28 ± 4	27 ± 2
L-Ann, 1.5 μg/ml	35 ± 1	35 ± 5	27 ± 1	33 ± 1	37 ± 1	30 ± 1	33 ± 2	33 ± 3	34 ± 5
Dx, 0.12 μg/ml	75 ± 2	11 ± 4	14 ± 5	82 ± 1	12 ± 1	6 ± 1	68 ± 3	23 ± 3	9 ± 0
Dx, 0.36 μg/ml	48 ± 1	7 ± 1	45 ± 1	56 ± 6	9 ± 1	35 ± 5	55 ± 10	15 ± 0	30 ± 10
MDA-MB-435									
Control	49 ± 5	32 ± 1	19 ± 1	54 ± 9	27 ± 5	19 ± 4	50 ± 1	30 ± 0	20 ± 1
L-Ann, 0.03 μg/ml	35 ± 3	16 ± 5	49 ± 2	43 ± 3	21 ± 5	36 ± 2	54 ± 3	17 ± 7	29 ± 3
L-Ann, 0.1 μg/ml	5 ± 1	15 ± 1	80 ± 6	13 ± 3	8 ± 1	79 ± 1	16 ± 5	35 ± 4	49 ± 2
Dx, 0.12 μg/ml	11 ± 6	17 ± 8	72 ± 13	29 ± 0.7	25 ± 1.5	46 ± 2	37 ± 16	22 ± 3	41 ± 14
Dx, 0.48 μg/ml	3 ± 1.5	66 ± 10	31 ± 9	1 ± 0.7	8 ± 7	91 ± 7	1 ± 0.7	19 ± 9	80 ± 10

[a]Data represent mean values ± SD from three independent experiments.

treatment with the IC_{50} concentration resulted in G_2M accumulations similar to those induced by the equitoxic concentration of the drug in MCF7 cells, whereas the maximum peak of cells (80%) in the G_2M phase was observed after exposure to the L-Ann IC_{80} concentration. Such an accumulation was stable until 48 h and then the cells began to reenter the cell cycle. Cell cycle effects induced by the IC_{50} concentration of Dx were similar to those induced by the IC_{80} concentration of L-Ann. After exposure to the IC_{80} Dx concentration, cells predominantly accumulated in the S phase at 24 h, then moved from S to G_2M, where 80% were still blocked after 72 h.

Effects of L-Ann and Dx on Proteins Involved in G_2 to M Transition Regulation

Since L-Ann and Dx mainly induced alteration in cell progression through G_2M phases, kinase activity of cyclin B1-associated cdc2 was measured on the substrate histone H1 after different treatments. In MCF7 cells (Fig. 2), exposure to the IC_{50} L-Ann concentration caused an inhibition of cdc2 kinase activity (−30%) in correspondence with the maximum accumulation of cells in G_2M (59%) at 24 h from the beginning of treatment. Then, as cells slowly reentered the cell cycle, cdc2 kinase activity increased to a level similar to that of control. Moreover, treatment of cells with the IC_{80} L-Ann concentration induced a stable suppression of cdc2 kinase activity (from −57 to −98% with respect to control) over 72 h that paralleled the permanent block of cells in the G_2M phase. Exposure of cells to the IC_{50} Dx concentration (which caused a permanent $G_{0/1}$ cell block) induced a progressive decrease of cdc2 kinase activity for 72 h (from −24 to −81%). Treatment with the IC_{80} Dx concentration caused a marked reduction in histone H1 phosphorylation after 72 h (−72%), the time point at which cells were still accumulated in G_2M.

Western blot analysis performed on treated MCF7 cells (Fig. 3) showed that the inhibition of cdc2 kinase activity observed with the highest concentration of L-Ann and both concentrations of Dx were accompanied by a marked reduction, or a complete disappearance, of cyclin B1 and a low level of cdc2 expression. Reduction in the extent of cdc2 phosphorylation on Tyr15 residue was also observed.

As shown in Figure 4, in MDA-MB-435 cells treated with L-Ann and Dx at all concentrations, kinase activities of cyclin B1-associated cdc2 were always higher than those of control. Superimposable results were obtained by directly immunoprecipitating cdc2 instead of cdc2 associated to cyclin B1 (data not shown). At a molecular level (Fig. 5), a slight increase in cdc2 and a marked increase in cyclin B1 expression was observed after different treatments at nearly all times considered. Moreover, cdc2 appeared strongly phosphorylated.

Induction of Apoptosis by L-Ann and Dx

To evaluate whether a different ability to induce programmed cell death could account for the different cytotoxic activity observed

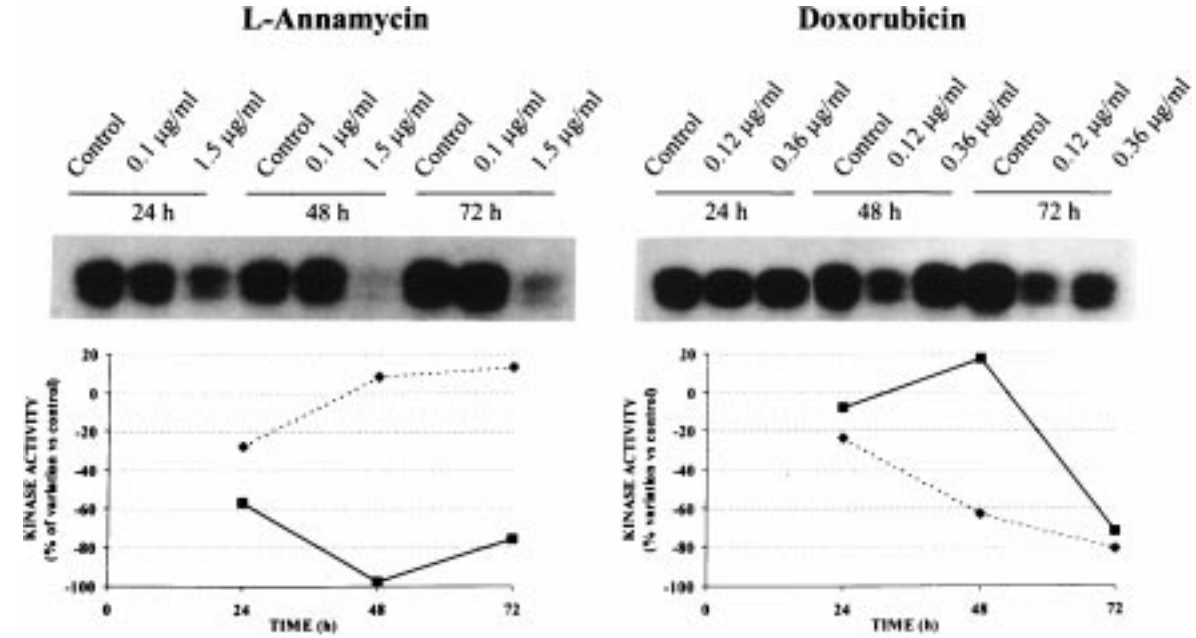

Fig. 2. Effect of L-Ann and Dx on cdc2 kinase activity in MCF7 cells. Cells were incubated with solvent (control) or with IC_{50} (–◆–) and IC_{80} (–■–) concentrations of L-Ann and Dx for 4 h. At the end of the treatment, cells were incubated for an additional 20, 44, and 68 h in drug-free medium (indicated as 24, 48,72 h from the beginning of drug treatment). Immunoprecipitation and kinase assay were performed as described in Materials and Methods. Following autoradiography, reactions were quantified by densitometry. Kinase activities of treated samples were expressed as percentage variation with respect to kinase activities of individual controls. Autoradiography of phosphorylated histone H1 and results of quantification of cdc2 kinase activity are shown.

after treatment of the two cell lines with L-Ann and Dx, we determined the occurrence of apoptosis by considering several end points. Evaluations were carried out separately in floating and adherent cells. However, since significant numbers of apoptotic cells were detected only in the floating fraction, only results on floating cells are reported. Figure 6 shows the percentage of cells with an apoptotic morphology, determined under fluorescence microscopy after staining of cells with propidium iodide. Only treated samples with at least a five-fold increase in the percentage of apoptotic cells with respect to controls were considered significantly different from untreated controls.

In the MCF7 cell line, about 10% (from 8 to 12%) of control floating cells showed an apoptotic morphology. No appreciable differences were found in the fraction of apoptotic cells after L-Ann or Dx treatment. In the MDA-MB-435 cell line, control floating cells showed only a negligible percentage of cells with an apoptotic morphology (from 1.5 to 3%). Such a percentage markedly increased in samples treated with the highest L-Ann concentration (18–20%) and with both Dx concentrations (10–20%). Superimposable results regarding the presence of apoptotic cells were obtained by flow cytometric analysis of samples treated with L-Ann and Dx and evaluated 72 h after the beginning of treatment (Fig. 7). Specifically, in MCF7 cells, small and similar pre-G_1 apoptotic cell peaks were observed in control and treated samples. Conversely, in MDA-MB-435 cells, no hypodiploid DNA peaks appeared in control cells, whereas marked pre-G_1 apoptotic cell peaks were observed in treated samples. Similar percentages of cells with hypodiploid DNA content were found after treatment with the two drugs: from 16 to 28% for L-Ann and from 21 to 23% for Dx.

Gel electrophoresis analysis of DNA from floating cells (Fig. 8) did not evidence the presence of internucleosomal DNA fragmentation, one of the typical features of apoptosis, in MCF7 cells treated with L-Ann at any concentration or time considered. Conversely, in MDA-MB-435 cells a dose-dependent induction of the DNA ladder was evident after treatment with L-Ann. Superimposable results were obtained with Dx (data not shown).

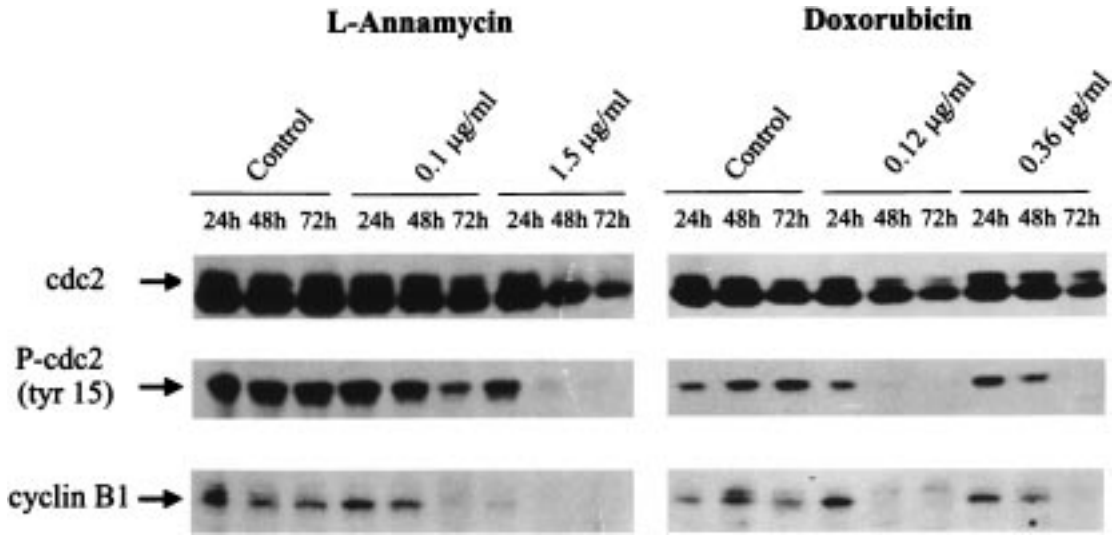

Fig. 3. A representative experiment illustrating the effect of L-Ann and Dx on the expression of proteins involved in the control of the G_2 checkpoint in MCF7 cells. Cells were incubated with solvent (control) or with IC_{50} and IC_{80} concentrations of L-Ann and Dx for 4 h. At the end of the treatment, cells were incubated for an additional 20, 44 and 68 h in drug-free medium (indicated as 24, 48, 72 h from the beginning of drug treatment). Western blots, performed as described in Materials and Methods, were probed with monoclonal antibodies for cdc2, phospho-specific cdc2 (Tyr 15), and cyclin B1.

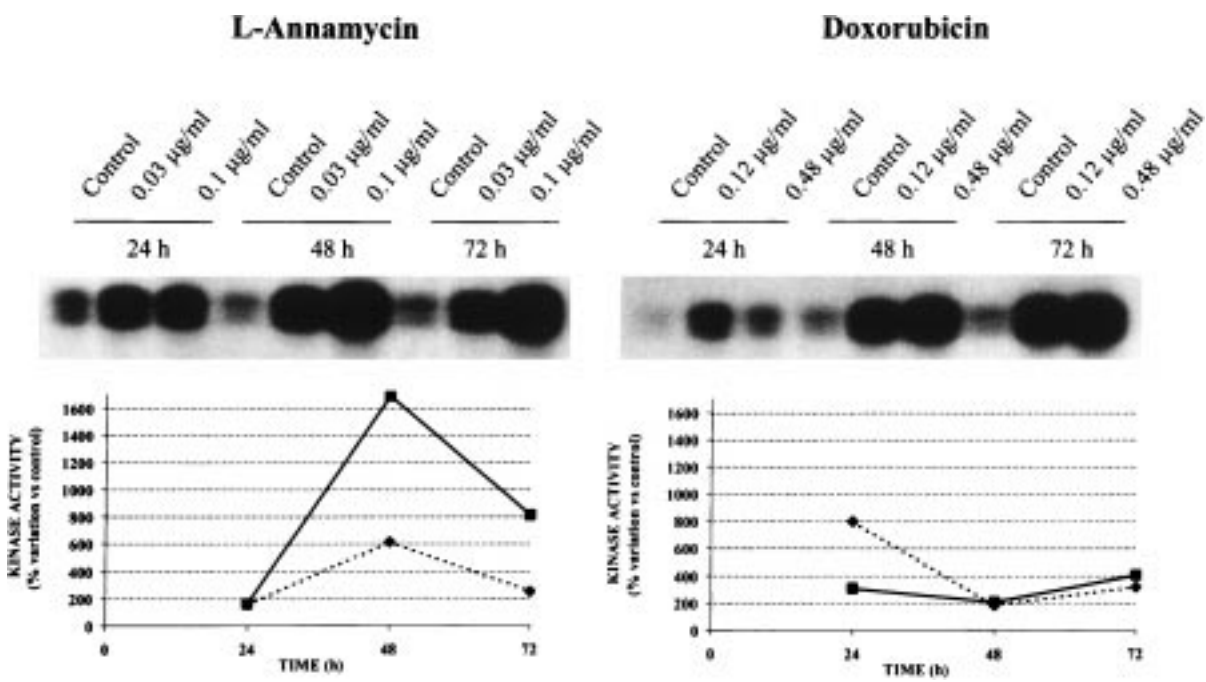

Fig. 4. Effect of L-Ann and Dx on cdc2 kinase activity in MDA-MB-435 cells. Cells were incubated with solvent (control) or with IC_{50} (–◆–) and IC_{80} (–■–) concentrations of L-Ann and Dx for 4 h. At the end of the treatment, cells were incubated for an additional 20, 44, and 68 h in drug-free medium (indicated as 24, 48, 72 h from the beginning of drug treatment). Immunoprecipitation and kinase assay were performed as described in Materials and Methods. Following autoradiography, reactions were quantified by densitometry. Kinase activities of treated samples were expressed as percentage variation with respect to kinase activities of individual controls. Autoradiography of phosphorylated histone H1 and results of quantification of cdc2 kinase activity are shown.

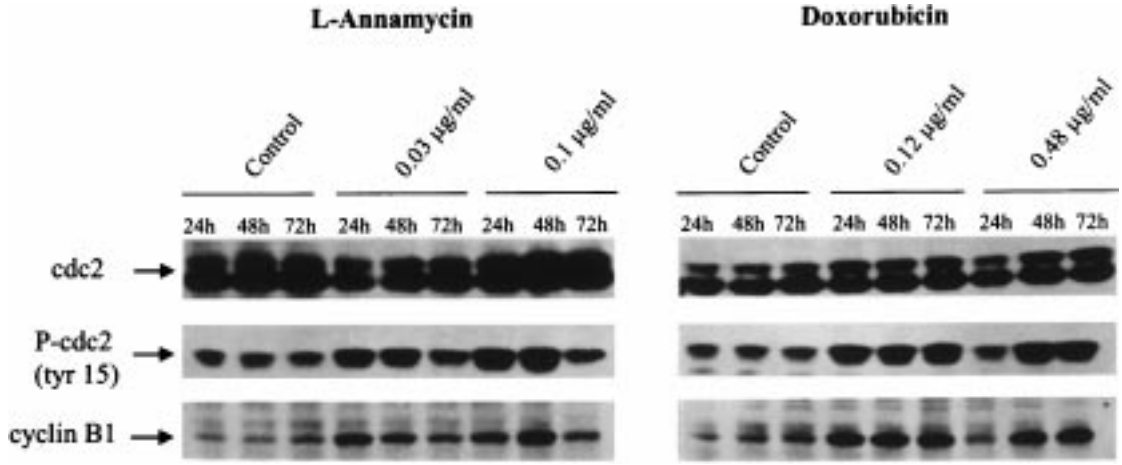

Fig. 5. A representative experiment illustrating the effect of L-Ann and Dx on the expression of proteins involved in the control of the G_2 checkpoint in MDA-MB-435 cells. Cells were incubated with solvent (control) or with IC_{50} and IC_{80} concentrations of L-Ann and Dx for 4 h. At the end of the treatment, cells were incubated for an additional 20, 44, and 68 h in drug-free medium (indicated as 24, 48, 72 h from the beginning of drug treatment). Western blots, performed as described in Materials and Methods, were probed with monoclonal antibodies for cdc2, for phospho-specific cdc2 (Tyr 15) and cyclin B1.

Effects of L-Ann and Dx on the Expression of Proteins Involved in the Control of Apoptosis

In the wt p53 cell line MCF7, L-Ann and Dx induced a similar dose-dependent increase in p53 protein with concomitant marked induction of $p21^{waf1}$protein and a slight increase in bax (Fig. 9). In MDA-MB-435 cells, which are characterized by a mutated p53 gene, L-Ann and Dx did not modify the expression of p53 or bax proteins, whereas a time- and dose-dependent upregulation of $p21^{waf1}$ was observed (Fig. 10). Moreover, a decrease of bcl-2 expression was detected in MCF7 cells after exposure to the highest L-Ann concentration and in MDA-MB-435 cells after treatment with both drug concentrations (Figs. 9 and 10).

DISCUSSION

Ann is a lipophilic anthracycline analogue specifically designed to be less cardiotoxic than Dx and to have high activity against tumors with an MDR phenotype. Most of the previously published studies addressed such issues. Since no investigation has been carried out to study the effects of Ann on cell cycle progression and to evaluate the induction of apoptosis by the drug in experimental models of human solid tumors, we focused our attention on such aspects in two human breast cancer cell lines, treated with a liposomal formulation of Ann.

At first we compared the cytotoxic activities of F-Ann, L-Ann, and Dx. We found that Ann was more active in the liposome-entrapped form than in the free form. Such a finding could be related to the low solubility of F-Ann in a culture medium after DMSO resuspension as well as to the higher cytotoxic potency conferred to Ann by liposome entrapment, as already reported [Zou et al., 1994]. In our system, L-Ann was more cytotoxic than Dx in both cell lines. Kolonias et al. [1999] found that, in parental Friend leukemia murine cells, Ann and Dx had similar cytotoxic activity. Conversely, Perez Soler et al. [1994, 1997] reported that, generally, in parental Dx-sensitive cells, Dx displayed higher activity than Ann. The different results could be explained on the basis of intrinsic differences in cell lines and assays used to measure cytotoxicity.

As regards drug-induced cell cycle perturbation, treatment with equitoxic L-Ann concentrations (IC_{50}) induced similar G_2M cell accumulations in MCF7 and MDA-MB-435, whereas exposure to IC_{80} L-Ann concentrations caused a marked and persistent accumulation of cells in G_2M only in the cell line with higher sensitivity to L-Ann, MDA-MB-435. Conversely, in MCF7 cells, the extent of cell cycle distribution was not dependent on drug concentration. In fact, only a slight, but stable increase in the G_2M cell fraction was detected after treatment with the highest L-Ann con-

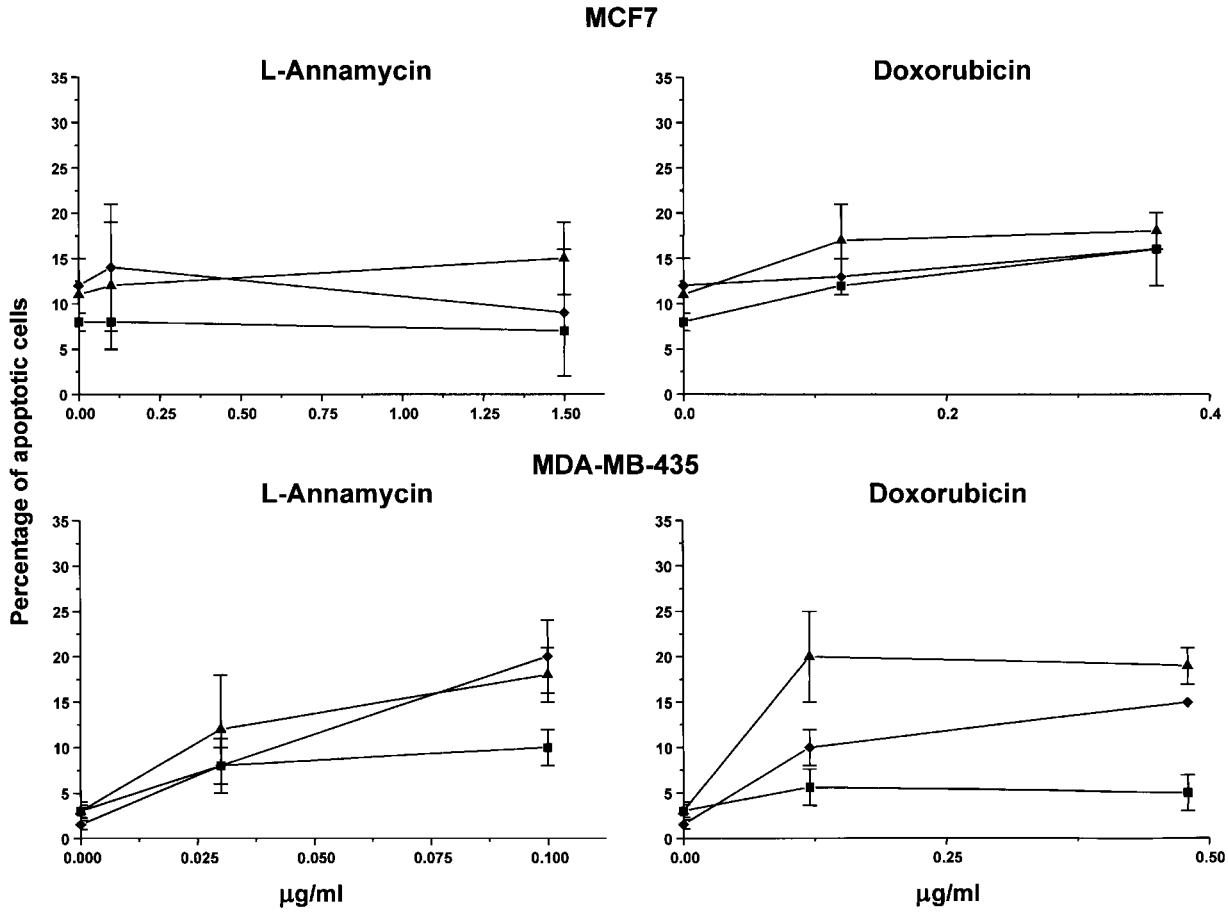

Fig. 6. Induction of apoptosis by L-Ann and Dx in MCF7 and MDA-MB-435 cells. Cells were exposed to each drug for 4 h, washed and incubated in drug-free medium for an additional 20 (■), 44 (◆), and 68 h (▲). Floating cells were then collected, stained with propidium iodide and viewed under the fluorescence microscope. Data represent mean values ± SD from three independent experiments.

centration. Although MCF7 and MDA-MB-435 displayed similar sensitivity to Dx, different cell cycle perturbations were induced. In MCF7, Dx caused a G_2M phase accumulation only after treatment with the highest concentration, whereas the IC_{50} Dx induced a marked block of cells in $G_{0/1}$. A similar cell cycle effect was already observed after treatment of MCF7 cells with epidoxorubicin, a Dx analogue [Silvestrini et al., 1997]. Moreover, Fornari et al. [1996] found that treatment of MCF7 cells with Dx produced arrest in G_1 and G_2 phases. As regards MDA-MB-435 cells, we observed a dose-dependent accumulation of cells in G_2M after exposure to Dx. Overall, our results indicated that the different cytotoxic effect of L-Ann observed in MCF7 and MDA-MB-435 was related to the extent of cell cycle perturbation induced by the IC_{80} concentration of the drug. No correlation was found for Dx.

We investigated whether differences in cell cycle perturbation could be ascribed to differences in expression of proteins involved in control of the G_2 checkpoint. Since the major regulator of G_2 to M transition is the M phase-promoting factor (MPF), a complex consisting of the catalytic subunit cdc2 and of the regulatory subunit cyclin B1 [Lewin, 1990], we focused our attention on the expression level of the two proteins. Moreover, since activation of the MPF in mitosis is associated with modification of phosphorylation of its two subunits, particularly cdc2 [Coleman and Dunphy, 1994], we studied the extent of phosphorylation on Tyr 15 of cdc2 and its kinase activity on histone H1.

In MCF7 cells, L-Ann- and Dx-induced G_2M arrest was associated with inhibition of cdc2 kinase activity, decreased levels of cdc2 expression, and almost complete disappearance of cyclin B1. In these cells, the lack of cyclin B1

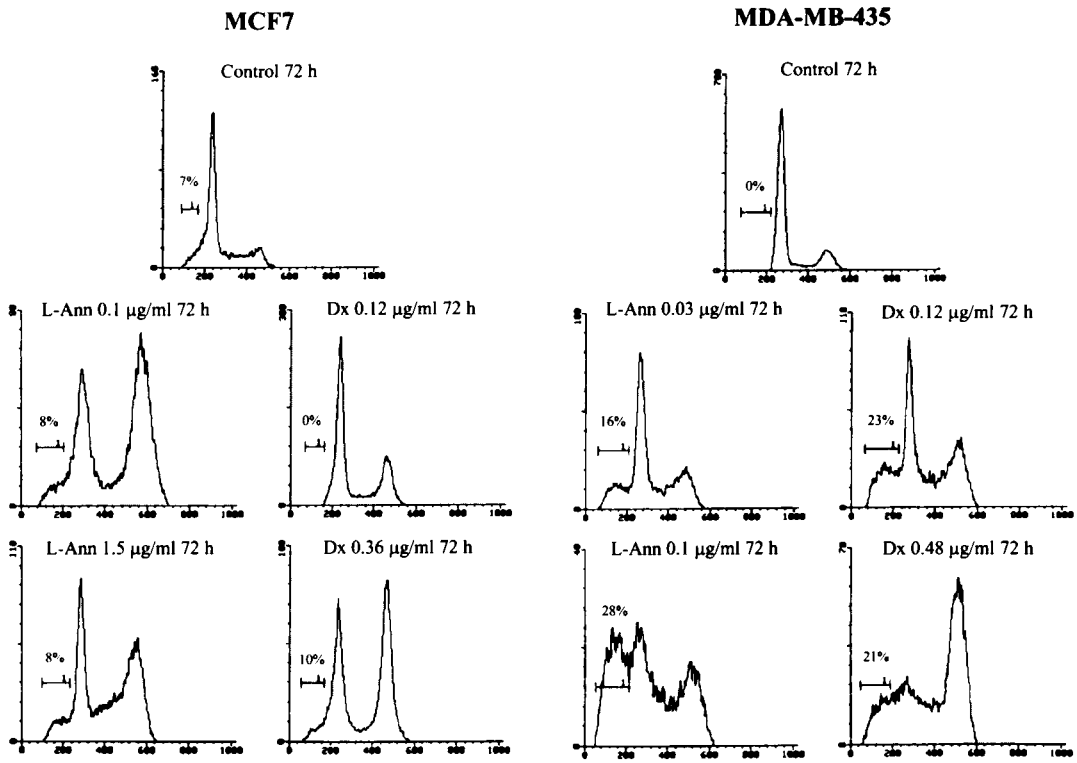

Fig. 7. Cell cycle analysis of MCF7 and MDA-MB-435 cells at 72 h from the beginning of a 4-h treatment with Dx or L-Ann. Cells were stained with propidium iodide and analyzed with a FACScan flow cytometer. Cell cycle distribution was evaluated on DNA plots by CellFit software according to the SOBR model (Becton Dickinson). The percentages of pre-G_1 apoptotic populations are reported in the bottom left hand corner of each histogram.

appeared to be the major responsible factor for the permanence of cells in G_2M after treatment with L-Ann and Dx. As already reported by Hoffmann et al. [1994] for Ilmofosine in CA46 cells, a lack of cyclin B1 protein would result in MPF complex formation insufficient to promote entry of cells in mitosis. A recent report from Innocente et al. [1999], demonstrated that in SKOV3 cells transfected with a temperature-sensitive mutant p53, at the permissive temperature, wild-type p53 controlled the G_2 checkpoint by decreasing intracellular levels of cyclin B1 as a consequence of decreased activity of cyclin B1 promoter. Since the MCF7 cell line carried wild-type p53, the observed disappearance of cyclin B1 could be ascribable to a p53-dependent suppression of transcription after DNA damage induced by L-Ann and Dx. In accordance with our results, Ling et al. [1996] found that in synchronized p53-mutated P388 cells, Dx treatment induced a cell cycle arrest at G_2M and decreased kinase activity of cdc2. However, they found that Dx prevented dephosphorylation of cdc2 kinase and caused accumulation of cyclin B1, thus altering the function of the cdc2/cyclin B1 complex.

In MDA-MB-435 cells, G_2M arrest induced by L-Ann and Dx was accompanied by an activation of cdc2 kinase and an increase in cyclin B1 and cdc2 levels and phosphorylation. In the same cell line, Yu et al. [1998] found that the arrest of cells in the G_2M phase after treatment with taxol was associated with activation of cdc2 and induction of apoptosis. Treatment with the cdc2 inhibitor olomucine prevented taxol-induced apoptosis and cdc2 activation. Shi et al. [1994] have already reported that premature activation of cdc2 is required for apoptosis induction, and a link between unscheduled activation of cdc2 and programmed cell death was found not only in HL-60 cells after treatment with different drugs [Shimizu et al., 1995], but also in solid tumors after exposure to taxol [Donaldson et al., 1994; Zaffaroni et al., 1998]. Therefore, we hypothesized that the activation of cdc2 observed in MDA-MB-435 after treatment with L-Ann and Dx was related to the induction of apoptosis observed in cells blocked in G_2M.

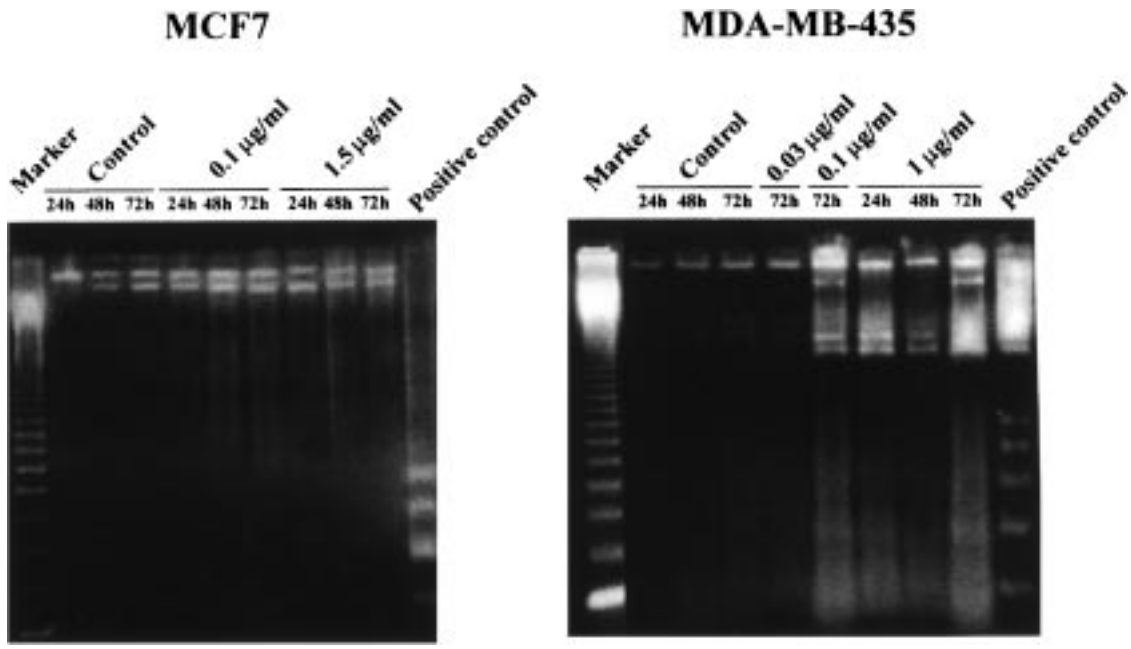

Fig. 8. Electrophoretic pattern of DNA extracted from MCF7 and MDA-MB-435 floating cells after 24, 48, and 72 h from the beginning of a 4-h treatment with different concentrations of L-Ann. HL-60 cells treated with 30 µg/ml of VM26 for 8 h were used as positive control of apoptosis induction.

The increased level of cdc2 kinase activity in the presence of a hyperphosphorylated cdc2 could be due to the fact that only cdc2 phosphorylation on Tyr 15 was detected in the present study, whereas two phosphorylation sites displaying different functions are present in the ATP-binding site of cdc2. Borgne and Meijer [1996] found that in synchronized starfish oocytes, dephosphorylation of cdc2 occurred in two steps: Thr 14 dephosphorylation was followed by Tyr 15 dephosphorylation. The intermediate form of cdc2, dephosphorylated

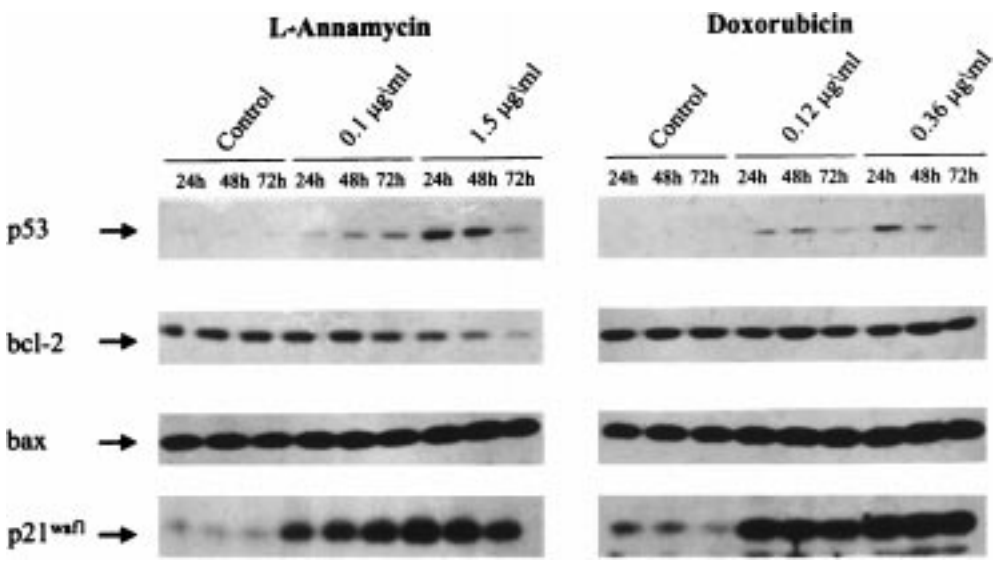

Fig. 9. A representative experiment illustrating the effect of L-Ann and Dx treatment on the expression of proteins involved in the control of apoptosis in MCF7 cells. Cells were incubated with solvent (control) or with IC_{50} and IC_{80} concentrations of L-Ann and Dx for 4 h. At the end of the treatment, cells were incubated for an additional 20, 44, and 68 h in drug-free medium (indicated as 24, 48, 72 h from the beginning of drug treatment). An aliquot of 100 µg of whole-cell extract was separated and electrophoretically blotted. Western blots, performed as described in Materials and Methods, were probed with antibodies for p53, bcl-2, bax, and $p21^{waf1}$.

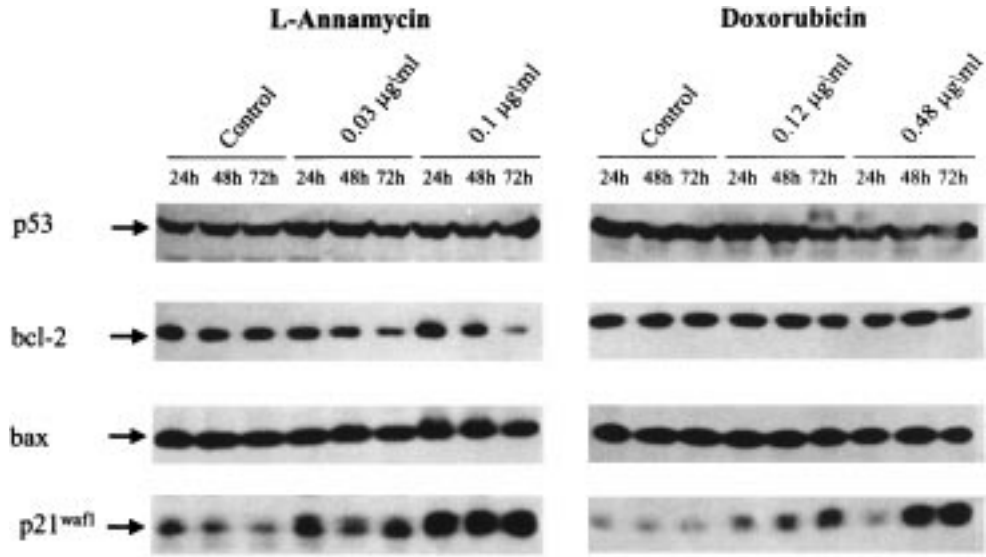

Fig. 10. A representative experiment illustrating the effect of L-Ann and Dx treatment on the expression of proteins involved in the control of apoptosis in MDA-MB-435 cells. Cells were incubated with solvent (control) or with IC_{50} and IC_{80} concentrations of L-Ann and Dx for 4 h. At the end of the treatment, cells were incubated for an additional 20, 44, and 68 h in drug-free medium (indicated as 24, 48, 72 h from the beginning of drug treatment). An aliquot of 100 μg of whole-cell extract was separated and electrophoretically blotted. Western blots, performed as described in Materials and Methods, were probed with antibodies for p53, bcl-2, bax, and $p21^{waf1}$.

on Thr 14 and phosphorylated on Tyr 15 (the same form that was detected in our study), displayed significant kinase activity, showing that the Thr 14 residue carried more powerful inhibitory potential than the Tyr 15 residue.

As regards apoptosis, a similar induction of programmed cell death was evidenced after treatment with L-Ann and Dx only in the p53-mutated cell line MDA-MB-435. In MCF7, a low percentage of cells with an apparent apoptotic morphology and hypodiploid DNA content was similarly detected in control and treated cells. Although several papers have reported that MCF7 cells are able to undergo apoptosis following different stimuli [Shao et al., 1995; Wang and Phang, 1995], our findings are consistent with those of Fornari et al. [1996], who failed to observe apoptosis after treatment of MCF7 cells with Dx, as evidenced by the absence of cells with apoptotic morphology and DNA fragmentation. The absence of apoptosis after L-Ann and Dx treatment in MCF7 cells could be explained by the hypothesis that various intrinsic cytoprotective mechanisms might be present in the cells, such as high endogenous protein kinase C activity [Jarvis et al., 1994], matrix adherence [Boudreau et al., 1995], or absence of bcl-x_s [Sumantran et al., 1995].

We also investigated whether the different susceptibility of MCF7 and MDA-MB-435 cells to undergo apoptosis might be explained by a different expression of proteins involved in the control of apoptotic cell death. In both cell lines, treatment with L-Ann or Dx caused a marked induction of the cyclin-dependent kinase inhibitor $p21^{waf1}$ [Xiong et al., 1993]. It is well known from the literature that $p21^{waf1}$ is a transcription target of p53 and is strongly induced by DNA damage in cells expressing functional p53 [El-Deiry et al., 1994]. However, its activation can also occur independently of p53 [Russo et al., 1995]. At present, the actual role of $p21^{waf1}$ in the control of apoptosis still remains controversial and appears dependent on the cell context. In fact, although $p21^{waf1}$ has been demonstrated to be involved in the induction of apoptosis in certain tumor cell models [Sheikh et al., 1995], it has also been suggested that $p21^{waf1}$ contributes to the ability of tumor cells to endure stress and exerts a protective function on tumor cell survival [Gorospe et al., 1997]. As regards bax, only a slight increase was observed in MCF7 cells after drug treatment, whereas a decrease in bcl-2 protein expression was found after treatment with L-Ann in both cell lines. Overall, no differences in expression of the major proteins involved in apoptosis could

account for the different susceptibility of the two cell lines to undergo apoptosis after L-Ann and Dx treatment.

In conclusion, our results indicate that L-Ann is more cytotoxic than Dx in breast cancer cell lines. However, this higher cytotoxicity cannot be explained by differences in cell cycle effects and apoptosis induction. Such findings suggest the opportunity to carry out further studies for the identification of possible preferential targets of L-Ann in human tumor cells in view of the clinical use of the new anthracycline.

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