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ORIGINAL ARTICLE

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Induction of DNA damage, inhibition of DNA synthesis and suppression of c-*myc* expression by the anthracycline analog, idarubicin (4-demethoxy-daunorubicin) in the MCF-7 breast tumor cell line

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Abstract *Purpose*: Studies were designed to elucidate the basis for the antiproliferative activity of the anthracycline antibiotic, idarubicin (4-demethoxy-daunorubicin) in MCF-7 breast tumor cells. *Methods*: Growth inhibition was evaluated using the MTT tetrazolium dye assay, induction of DNA strand breaks was determined by alkaline elution, inhibition of DNA synthesis was assessed by measuring the incorporation of labelled thymidine into DNA, modulation of the expression of the c-*myc* oncogene was determined by Northern blotting and the induction of apoptosis was evaluated by alkaline unwinding, static field gel electrophoresis, terminal end labelling and assessment of cell morphology. *Results*: MCF-7 cells were relatively sensitive to idarubicin, with an IC_{50} value for growth inhibition of approximately 0.01 μM. While DNA strand breakage was not evident below a concentration of 0.1 μM idarubicin, where growth inhibition exceeded 70%, both the inhibition of DNA synthesis and suppression of c-*myc* expression closely paralleled the profile of antiproliferative activity for idarubicin. Finally, while exposure to idarubicin resulted in a substantial loss of viable cells within 48–72 h, there was no morphological evidence of apoptotic body formation. The absence of apoptosis in cells exposed to idarubicin was supported by studies demonstrating the absence of DNA fragmentation using gel electrophoresis, alkaline elution and in situ DNA end-labelling assays. *Conclusions*: The results of these studies extend previous results from this laboratory indicating an association between suppression of c-*myc* expression, inhibition of DNA synthesis and growth arrest by topoisomerase II inhibitors, as well as the lack of induction of apoptotic cell death by topoisomerase II inhibitors in MCF-7 breast tumor cells.

Key words Idarubicin · c-*myc* · Breast tumor cells

Abbreviations *DMEM* Dulbecco's modified Eagle's medium · *GAPDH* glyceraldehyde phosphate dehydrogenase · *m-AMSA* 4′-(9-acridinylamino) methane sulfon-m-anisidide · *MTT* 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide · *PBS* phosphate-buffered saline · *SDS* sodium dodecyl sulfate · *VM-26* teniposide, 4′-demethylepipodophyllotoxin-4-(4,6-0-thenylidene-beta-D-glucopyranoside

Introduction

The anthracycline antibiotics, which include the drugs doxorubicin and daunorubicin, have broad utility in the treatment of a variety of malignancies. Doxorubicin is one of the primary agents utilized in the treatment of breast cancer [18]. The 4-demethoxy derivative of daunorubicin, idarubicin, which can be administered orally [15], and which has been reported to circumvent multidrug resistance [2, 35], is used primarily against leukemias [5] but has also shown utility in the treatment of breast cancer [25, 32].

One of the primary mechanisms thought to mediate the antiproliferative and cytotoxic effects of drugs such as the anthracycline antibiotics is the inhibition of the religation activity of the enzyme, topoisomerase II [38], which results in the induction of strand breaks in DNA [16]. Previous work from this laboratory has demonstrated a lack of correspondence between the induction of DNA damage and the antiproliferative activity of these agents [3, 11, 14, 27, 43]. In contrast, inhibition of DNA synthesis has been shown to correlate closely with growth inhibition in both H-35 rat hepatoma and MCF-7 breast tumor cells [3, 11, 14, 27]. In addition,

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it has been reported from this laboratory that the topoisomerase II inhibitors, doxorubicin, VM-26 and m-AMSA, as well as ionizing radiation, are capable of suppressing the expression of the oncogene, c-*myc*, in the MCF-7 breast tumor cell line [3, 11, 14, 41]. Furthermore, the concentration-dependent suppression of c-*myc* expression has been determined to be predictive of growth arrest [3, 11, 14, 41]. Finally, it has been reported from this laboratory [10, 12, 41], as well as by other investigators [28, 34, 42] that MCF-7 breast tumor cells are generally refractory to apoptotic cell death induced by agents that damage DNA or otherwise perturb DNA integrity.

The purpose of the present work was to extend these previous findings to idarubicin by assessing the relationship between the effects of idarubicin on DNA synthesis, c-*myc* expression and growth of MCF-7 breast tumor cells. In addition, this work was designed to provide further evidence that cell death can occur in these breast tumor cells in the absence of oligosomal DNA fragmentation and apoptosis. The findings support the possibility that regulation of c-*myc* expression could be linked to the growth regulatory/growth arrest pathways in MCF-7 breast tumor cells.

Materials and methods

Materials

Dulbecco's modified eagle's medium (DMEM) was obtained from Hazelton Research Products, Denver, P. L-Glutamine, penicillin/streptomycin (10 000 U/ml penicillin and 10 mg/ml streptomycin), and fetal bovine serum were obtained from Whittaker Bioproducts, Walkersville, Md. Defined bovine calf serum was obtained from Hyclone Laboratories, Logan, Utah. Trypsin-EDTA (10 × 0.5% trypsin, 5.3 m*M* EDTA) was obtained from Gibco Laboratories, Grand Island, N.Y. Idarubicin (4-demethoxy-daunorubicin) was generously provided by Pharmacia/Adria Laboratories, Milwaukee, Wis. Idarubicin was dissolved in water and maintained as a frozen stock solution. Drug was diluted in incubation medium on the day of the experiment.

The radiolabelled compounds [^{3}H]thymidine (75 Ci/mmol), and [α-^{32}P]dCTP (3000 Ci/mmol) were obtained from DuPont NEN Research Products, Boston, Mass., respectively. Nuclease S_1 was obtained from Pharmacia LKB Biotechnology, Piscataway, N.J. Dimethyl sulfoxide, proteinase K, MTT, thymidine, and trichloroacetic acid and RNAse A were obtained from Sigma Chemical Co, St. Louis, Mo. Tetrapropyl ammonium hydroxide was obtained from Kodak Chemicals, (Rochester, N.Y.). Agarose was obtained from GIBCO BRL (Gaithersburg, Md.). All other chemicals were reagent or molecular grade, as appropriate.

The c-*myc* probe, an *Eco*RI/*Cla*I fragment of pMC41 3RC containing the third exon of the human c-*myc* gene was kindly provided by Dr. Eric Westin of the Medical College of Virginia. Genomic or cDNA probes for glyceraldehyde-3-phosphate dehydrogenase (pHcGAP) were obtained from ATCC, Rockville, Md.

Cell line

The MCF-7 breast tumor cell line was kindly provided by the laboratory of Dr. Kenneth Cowan at the National Cancer Institute, Bethesda, Md, and cells were maintained as monolayers in DMEM supplemented with glutamine (0.292 mg/ml), penicillin/streptomycin (0.5 ml/100 ml medium), 5% fetal bovine serum, and 5% defined bovine serum at 37 °C in an atmosphere containing 5% CO_2.

Cell proliferation and cell death

Growth Inhibition

The capacity of idarubicin to interfere with the growth of the MCF-7 breast tumor cells was determined using the MTT tetrazolium dye assay, as described in detail previously [11]. Briefly, cells subcultured at a density of 1×10^4 cells/ml in 96-well microplates (Costar, Cambridge, Mass.) were incubated with various concentrations of idarubicin for 2 h. Drug was aspirated, cells were washed with incubation medium, and permitted to grow for an additional 72 h prior to determination of viable cell numbers.

Cell killing

The capacity of idarubicin to produce cell death was determined by monitoring the decline in the number of cells originally plated using trypan blue exclusion and cell counting. MCF-7 cells subcultured at a density of 1.5×10^4 cells/ml in 25-cm^2 T flasks (Costar) were incubated with 0.5 μM idarubicin for 2 h at 37 °C. Drug was aspirated and the cells were washed with ice-cold phosphate-buffered saline (PBS, pH 7.4). Cells were released from flasks by incubation with trypsin (0.05 mg/ml)/EDTA (0.02 mg/ml) for 5 min at 37 °C, collected in ice-cold PBS (pH 7.4), and centrifuged at 4 °C. Cell pellets were resuspended in 300 µl ice-cold PBS and aliquots were mixed with trypan blue. Cells were loaded on a hemocytometer and viable cell numbers determined (cell viability was assessed by exclusion of trypan blue dye).

Determination of DNA damage

Loss of integrity of bulk DNA was determined by enhanced-fluorescence alkaline unwinding analysis using either the alkaline unwinding procedure of Kanter and Schwartz [21], as described previously [11, 27]. For alkaline unwinding, cells in 75-cm^2 T flasks (Costar) were incubated with idarubicin for 2 h, washed with PBS (pH 7.4), released from flasks by incubation with 0.05 mg/ml trypsin in 0.02 mg/ml EDTA for 5 min at 37 °C, and collected in ice-cold PBS. Pelleted cells were resuspended in saline and subjected to timed alkaline denaturation in 0.1 *N* NaOH. Denaturation was terminated by neutralization in 0.1 *N* HCl. Cells were further diluted in PBS and lysed by the addition of 200 m*M* K_2HPO_4, 50 m*M* EDTA, 0.16% *N*-lauroylsarcosine with brief sonication. Damage to bulk DNA in cell lysates was quantified by spectrofluorophotometry in the presence of Hoechst-33258 (λex = 350, λem = 450). Induction of strand breaks was demonstrated by reduction in net DNA fluorescence. Values were standardized against graded DNA strand breakage induced by scaled [^{137}Cs] irradiation (0.3 to 30 Gy).

Alkaline unwinding was also used to determine whether DNA fragmentation may have occurred by assessing the integrity of DNA at 72 h after exposure to drug. We have previously demonstrated that DNA fragmentation assessed using this assay parallels the induction of apoptosis in HL-60 human leukemic cells [17, 20].

The induction of DNA damage by idarubicin was confirmed using the alkaline elution procedure of Kohn et al. [23]. Cells were prepared as for alkaline unwinding, and alkaline elution was performed as described in detail previously [27].

Influence of idarubicin on DNA biosynthesis

The effects of idarubicin on the rates of DNA synthesis were determined by monitoring the rate of incorporation of [^{3}H]thymidine into acid-precipitable material over a time course of 40 min as previously described [11, 27]. MCF-7 cells in 24-well plates (Costar)

were exposed to idarubicin for 2 h and washed with Hanks' buffered salts solution (pH 7.4; Whittaker Biochemicals, Walkersville, Md.) at room temperature prior to incubation with [^{3}H]thymidine for 40 min. At appropriate times, cells were washed with ice-cold PBS, lysed with trypsin/EDTA and precipitated with ice-cold trichloroacetic acid. The percentage inhibition of DNA biosynthesis was calculated from the relative rates of incorporation of the ^{3}H-labelled nucleic acid precursor into acid-precipitable material (on Millipore filters) in drug-treated versus untreated control cells.

Influence of idarubicin on expression of c-*myc* and GAPDH

After incubation with idarubicin for appropriate times and at stated concentrations, cells were washed twice with 10 ml ice-cold PBS (pH 7.4), and lysed in 4 *M* guanidine isothiocyanate and 0.5% sodium lauryl sarcosine. RNA was isolated by ultracentrifugation through a 5.7 *M* cesium chloride cushion at 41 000 *g* for 20 h at 20°C [7]. RNA was precipitated with 70% ethanol, RNA pellets were washed in 95% ethanol and 70% ethanol, and resuspended in Mili Q water.

RNA (10 μg) was denatured in 0.02 *M* morpholino propane sulfonic acid (pH 7.0), 5 m*M* sodium acetate, 1 m*M* EDTA, 2.2 *M* formaldehyde and 50% formamide. The samples were separated on a 6.6% formaldehyde/1% agarose gel [39]. Equal loading of RNA in each lane was confirmed by ethidium bromide staining. Blotting was carried out using Nytran transfer membranes (Schleicher & Schuell, Keene, N.H.).

Probes were radiolabelled using a nick translation kit from GIBCO BRL (Gaithersburg, Md.) and hybridized to blots in the presence of 50 m*M* sodium phosphate, pH 6.5, 5 × Denhardt's solution (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinyl pyrrolidine) 5 × SSC (0.75*M* NaCl, 0.075 *M* sodium citrate), 0.1% SDS, yeast RNA (250 μg/ml), 50% formamide and 10% dextran sulfate [39]. Hybridizations were for 16–20 h at 42 °C. Filters were washed three times at 42 °C for 5 min in 2 × SSC and 0.2% SDS followed by one wash in 2 × SSC and 0.2% SDS at 60 °C and one wash in 0.5 × SSC and 0.2% SDS for 40 min before autoradiography.

Qualitative analyses of DNA fragmentation by static field gel electrophoresis

The formation of oligonucleosomal DNA fragments (~0.2 to ~1.2 kbp) was assessed by conventional agarose gel electrophoresis as described previously [17, 20]. After drug exposure and washing, cells were released from flasks by incubation with trypsin (0.5 mg/ml)/EDTA (0.02 mg/ml) for 5 min, collected in ice-cold PBS at pH 7.4 and centrifuged at 1500 rpm for 5 min at 4 °C. Pelleted cells were resuspended in PBS and lysed by the addition of 5 m*M* Tris-base, 20 m*M* EDTA, 0.1% Triton X-100, pH 8.0 (yielding a final density of 2×10^7 cells/ml), and mixed thoroughly with gentle mechanical agitation; the lysates were then treated with proteinase-K (500 μg/ml; Sigma) at 55 °C for 18 h. The deproteinated extracts were centrifuged at 30 000 *g* for 45 min at 4 °C, and the pellets were discarded. The supernatants were treated with ribonuclease-A (100 μg/ml; Sigma) at 37 °C for 3 h. Aliquots of final lysate preparations (corresponding to 2×10^6 cells) were loaded into 2.25% agarose gels (Metaphor; FMC) impregnated with ethidium bromide (0.5 mg/ml). Low molecular weight DNA fragments were resolved by electrophoresis at 6.5 V/cm for 90 to 180 min in tris acetate/EGTA buffer. DNA fragments were visualized by UV transillumination. DNA molecular weight reference preparations (100 bp/step ladder; GIBCO-BRL) were run in parallel to facilitate estimation of DNA fragment size.

Morphological assessment of apoptosis

After exposure to idarubicin and trypsinization, pelleted cells were resuspended in PBS, fixed in conventional cytocentrifuge preparations, stained with 20% Wright-Giemsa stain, and the occurrence and mode of cell death in each treatment group was determined based on morphological criteria outlined previously [17, 20]. At least 300 cells were scored for each treatment by assessing the expression of cytoarchitectural characteristics of either apoptosis (cell shrinkage, condensation of nucleoplasm and cytoplasm, formation of membrane blebs and apoptotic bodies) or necrosis (cell swelling, nuclear expansion, deterioration of organellar membranes, gross cytolysis).

Terminal end labelling (TUNEL) assay

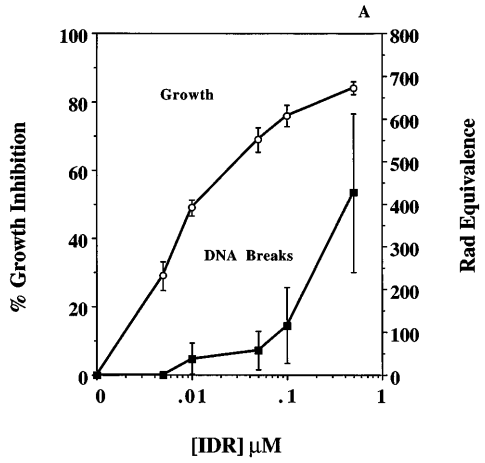
The method of Gavrieli et al. [13] was utilized as an independent assessment of apoptotic cell death. Briefly, following fixation in 4% formaldehyde, cells were treated with acetic acid/ethanol (1:2) for 5 min at −20 °C. The slides were then immersed in TDT buffer (30 m*M* Trizma base, pH 7.2, 140 m*M* sodium cacodylate, 1 m*M* cobalt chloride) containing 0.3 e.u./ml TdT (Boehringer-Mannheim) and fluorescein isothiocyanate-labelled dUTP (fluorescein-12-dUTP; Boehringer-Mannheim). The slides were incubated in a fully humidified atmosphere at 37 °C for 60 min. The reaction was terminated by washing the slides in PBS, after which they were counterstained with propidium iodide (5 μg/ml in 0.01% sodium citrate). After rinsing, the slides were examined under fluorescence utilizing an Olympus model BX-40 fluorescent microscope for cells exhibiting a bright green–yellow fluorescence indicative of apoptosis.

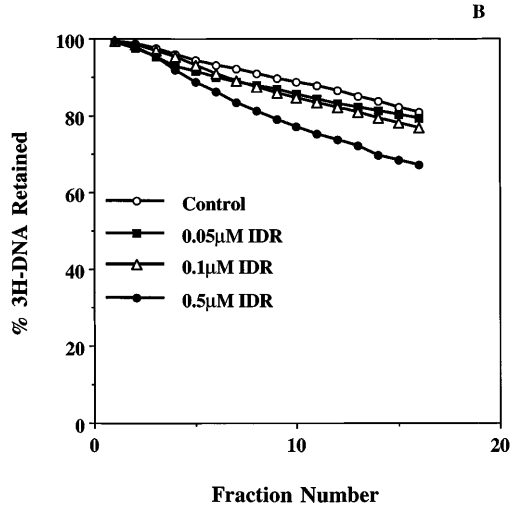
Results

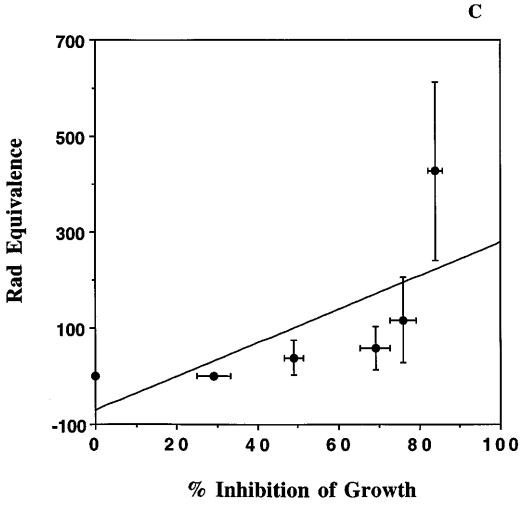
The influence of idarubicin on the proliferation of MCF-7 breast tumor cells was determined 72 h after acute drug exposure. Figure 1A indicates that idarubicin produced a concentration-dependent reduction in cell growth, with an IC_{50} value of approximately 0.01 μ*M*[1] The corresponding induction of DNA strand breaks after acute drug exposure was determined using the technique of alkaline unwinding. Figure 1A further demonstrates that there was little detectable DNA damage at the IC_{50}. Significant damage to DNA was evident only after cells were exposed to an idarubicin concentration of 0.5 μ*M*, where cell growth was inhibited by approximately 70%. These effects of idarubicin on DNA damage were confirmed using the alkaline elution assay. As shown in Fig. 1B, there was minimal DNA damage detected at idarubicin concentrations of 0.05 and 0.1 μ*M*. Significant DNA strand breakage was observed only at 0.5 μ*M* idarubicin. The plot presented in Fig. 1C demonstrates that the induction of DNA damage by idarubicin did not correspond with the extent of growth inhibition (correlation coefficient 0.47).

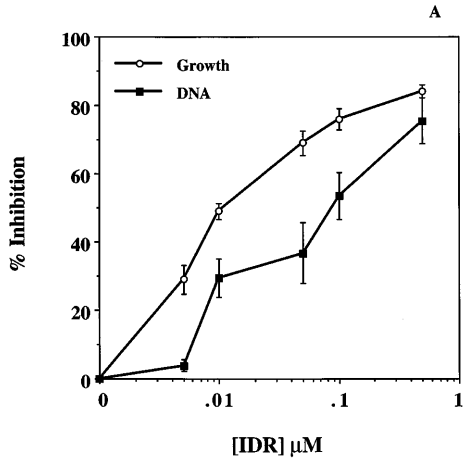
We have previously reported that growth inhibition by the topoisomerase II inhibitors, doxorubicin, VM-26 and m-AMSA, in MCF-7 cells [3, 11, 14] and by doxorubicin in H-35 rat hepatoma cells [27] corresponds closely with inhibition of DNA synthesis. Figure 2A demonstrates that idarubicin produced a concentration-dependent inhibition of DNA synthesis (assessed 2 h

[1] A similar dose-response relationship was generated with a 4-h exposure to idarubicin. These studies were performed to maintain consistency with the conditions for concentration-dependent effects of idarubicin on c-*myc* expression.









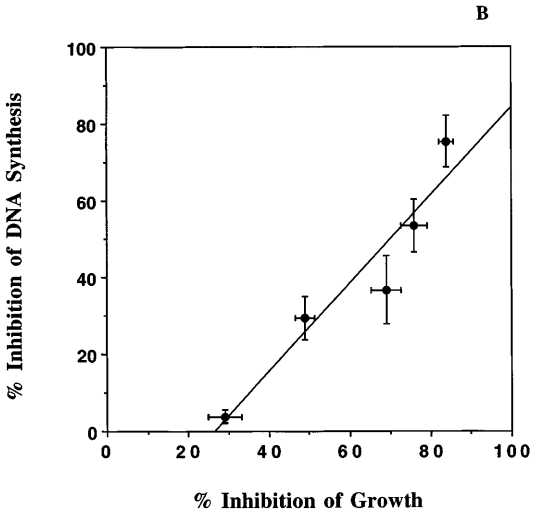


Fig. 2 A Growth inhibition and inhibition of DNA synthesis in MCF-7 breast tumor cells after acute exposure to idarubicin. MCF-7 cells were exposed to idarubicin for 2 h and growth arrest was assessed 72 h later by the MTT tetrazolium dye assay. Effects on DNA synthesis were assessed immediately after drug exposure and each point represents the mean ± standard error of five experiments. **B** Relationship between growth inhibition and suppression of DNA synthesis

after the initiation of drug exposure) which paralleled the inhibition of growth. Figure 2B indicates a correlation between the inhibitory effect of idarubicin on cell growth and on DNA synthesis (correlation coefficient 0.91).

Fig. 1A Growth inhibition and induction of DNA damage in MCF-7 breast tumor cells after acute exposure to idarubicin. MCF-7 cells were exposed to idarubicin for 2-h and growth arrest was assessed 72 h later by the MTT tetrazolium dye assay. DNA strand break induction was monitored immediately after the 2-h drug exposure by alkaline unwinding. Each point relating to growth inhibition represents the mean ± standard error of eight replicate experiments. Each point relating to induction of DNA strand breaks represents the mean ± standard error of four to six replicate experiments. **B** Induction of DNA damage in MCF-7 cells as measured by alkaline elution. In this representative experiment, MCF-7 cells were exposed to idarubicin for 2 h and DNA strand break induction was monitored immediately after the 2-h drug exposure. All samples were incubated with proteinase K in order to digest DNA–protein cross-links prior to elution. **C** Lack of correspondence between the induction of DNA strand breaks and growth inhibition by idarubicin (Data were taken from **A)**

One of the primary interests pursued in our laboratory has been to determine whether alterations in the expression of the oncogene, c-*myc*, could be an early event in the cellular response to topoisomerase II inhibitors. In this context, we have previously determined that early suppression of c-*myc* expression (i.e. within 2–4 h after drug exposure) is closely associated with growth arrest by VM-26, m-AMSA and doxorubicin [3, 12, 14]. Consequently, we evaluated the capacity of idarubicin to alter c-*myc* expression in a time- and concentration-dependent manner. Figure 3 presents a representative Northern blot as well as pooled data from four experiments and demonstrates a time-dependent suppression of c-*myc* expression in MCF-7 cells exposed to 0.5 μ*M* idarubicin. The transient increase in c-*myc* expression which was evident at 1 h in the Northern blot was observed only in a single experiment. Maximal suppression of c-*myc* expression by idarubicin occurred between 3 and 4 h. As shown, levels of the constitutively expressed housekeeping gene, GAPDH, were essentially unchanged in cells exposed to idarubicin.

Our previous findings relating to drug and radiation-induced suppression of c-*myc* expression [3, 12, 14, 41] suggest that c-*myc* expression could be closely associated with the growth arrest pathway which responds to DNA-damaging agents in this breast tumor cell line. Consequently, we were interested in determining whether a similar relationship is evident for idarubicin.

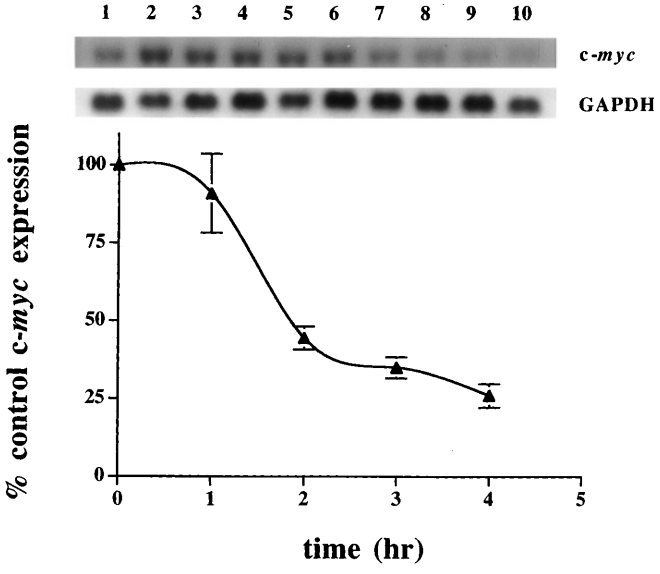


Fig. 3 *Upper* Representative Northern blot demonstrating the time-dependent reduction in c-*myc* expression in MCF-7 breast tumor cells after exposure to idarubicin and the lack of effect of idarubicin on GAPDH expression. (*lanes 1–5* control cells at 0, 1, 2, 3 and 4 h, respectively, after initiation of the study; *lanes 6–10* drug-treated cells at 0, 1, 2, 3 and 4 h after initiation of the study). *Lower* Pooled data indicating the time-dependent suppression of c-*myc* expression by idarubicin. Each point represents the mean ± standard error for c-*myc* expression (normalized for GAPDH expression) of four replicate experiments

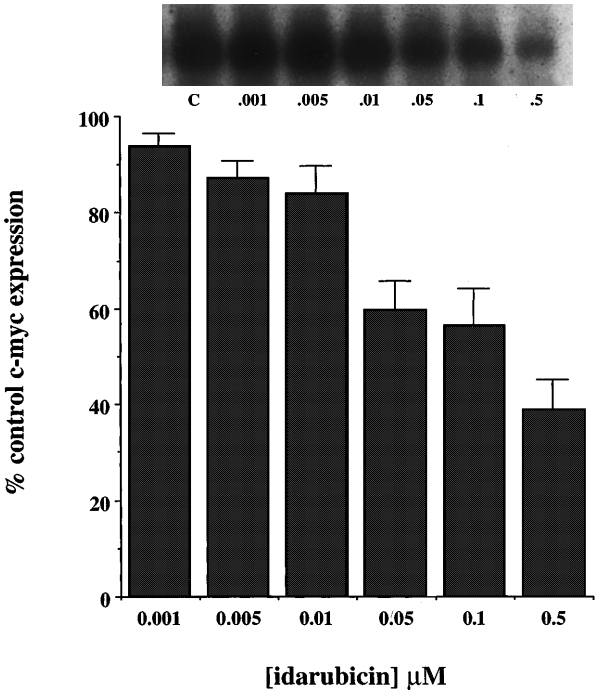


Fig. 4 *Upper* Representative Northern blot demonstrating the concentration-dependent reduction in c-*myc* expression at 4 h after treatment with the indicated concentrations of idarubicin. *Lower* Quantitative representation of idarubicin-induced suppression of c-*myc* expression. Each point represents the mean ± standard error for c-*myc* expression (normalized for GAPDH expression) of four replicate experiments

The effects of various concentrations of idarubicin on c-*myc* expression (at 4 h after drug exposure) were determined over the same concentration range used to assess growth inhibition. Figure 4 presents a representative Northern blot accompanied by pooled data from four independent experiments and shows a concentration-dependent reduction in c-*myc* expression by exposure to idarubicin; again, expression of GAPDH was essentially unchanged (not shown). Figure 5A demonstrates that the suppression of c-*myc* expression by idarubicin paralleled growth inhibition. Figure 5B indicates a correlation between the inhibitory effects of idarubicin on cell growth and on c-*myc* expression (correlation coefficient of 0.9).

The MTT assay, which was utilized to generate the growth inhibition data, does not discriminate between growth arrest and cell killing. In order to determine whether acute exposure to idarubicin resulted in cell death, cell numbers were monitored over a time period of 72 h after acute exposure of MCF-7 cells to idarubicin. Table 1 indicates that viable cell numbers were reduced by between 70% and 80% over 48–72 h following acute exposure to the drug.

Analysis of cell morphology at intervals of 24 h after drug treatment supports the concept that these breast tumor cells were not undergoing apoptotic cell death. As shown in Fig. 6, there was no evidence of cell shrinkage, nuclear condensation or apoptotic bodies in MCF-7 cells

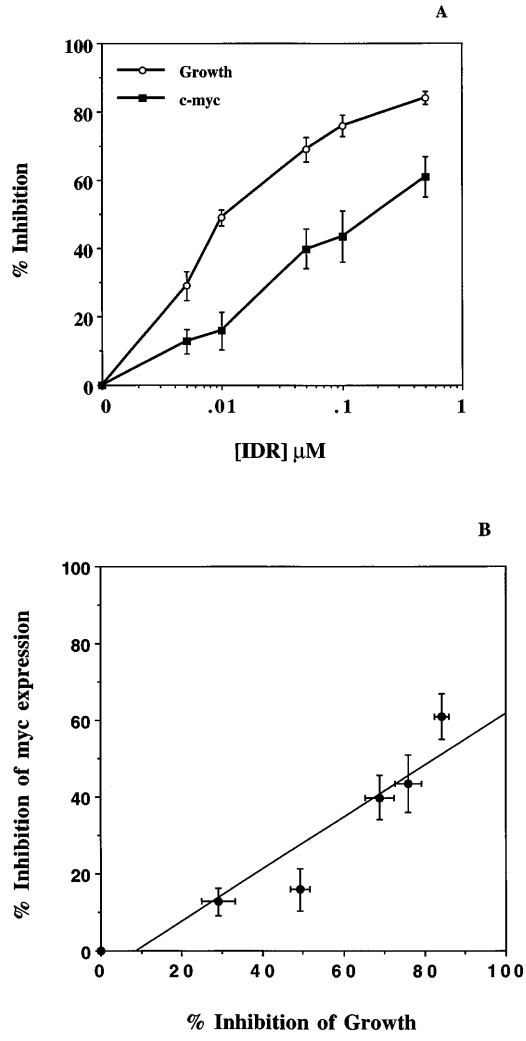


Fig. 5 A Growth inhibition and suppression of c-*myc* expression in MCF-7 breast tumor cells after acute exposure to idarubicin. MCF-7 cells were exposed to idarubicin for 2 h and growth arrest was assessed 72 h later by the MTT tetrazolium dye assay.[1]Effects on c-*myc* expression were assessed at 4 h after drug exposure and each point represents the mean ± standard error of four experiments. **B** Relationship between growth inhibition and suppression of c-*myc* expression

exposed to idarubicin. Analysis of DNA integrity by static field gel electrophoresis confirmed the absence of DNA fragmentation in cells acutely exposed to idarubicin (not shown). Assessment of DNA integrity at 72 h after drug exposure using the alkaline unwinding procedure also failed to provide any evidence for DNA fragmentation (not shown). Finally, as shown in Fig. 7, the in situ TUNEL assay for detection of DNA fragmentation failed to demonstrate an increase in the number of cells with damaged DNA, confirming the absence of apoptotic cell death in MCF-7 cells exposed to idarubicin.

Table 1 Time-dependent reduction in viable cell numbers after acute exposure of MCF-7 cells to 0.5 μ*M* idarubicin. Each value is the mean ± standard error of two or three replicate experiments. The values indicate the proportion of cells remaining viable as a percentage of the original population at time zero. These studies of cell killing should be clearly distinguished from the data presented in Fig. 1, which compares growth in control and drug-treated cells after 72 h (i.e. growth inhibition)

24 h	48 h	72 h
83 ± 7.4	35.8 ± 5.7	28.8 ± 12.2

Discussion

Induction of DNA strand breaks

Idarubicin, like other members of the family of anthracycline antibiotics, is thought to interfere with cell growth and to induce cell death by inhibiting the activity of the enzyme, topoisomerase II, which results in the induction of DNA strand breaks [43, 44]. However, the extent of DNA damage produced by idarubicin is relatively low in comparison with that of other topoisomerase II inhibitors, such as m-AMSA and VM-26 [3, 14]. The present studies are consistent with previous findings from this laboratory [43] as well as by other investigators demonstrating a lack of correspondence between DNA strand breaks (or DNA–protein cross-links) and toxicity of idarubicin [1, 4, 9, 44]. For example, Zwelling et al. [44] were unable to demonstrate a relationship between DNA–protein cross-linking and the cytotoxicity of idarubicin in HL-60 leukemic cells. Similarly, Ferrazzi et al. [9] have reported that DNA damage is essentially undetectable at the IC_{50} for idarubicin and that the induction of DNA damage fails to correspond directly with growth inhibition in mouse fibrosarcoma cells. Capranico et al. [4] did not detect DNA strand breaks in P388 leukemia cells at the IC_{50} of idarubicin. A series of studies by Belvedere et al. [1] also suggest a dissociation between DNA strand break induction and growth inhibition by idarubicin. These investigators [1] reported that the extent of DNA damage increases in the absence of a corresponding increase in growth inhibition at elevated drug concentrations, and conversely that growth inhibition increases without a perceptible increase in DNA strand break induction at low drug concentrations.

Cell killing in the absence of apoptosis

We as well as others have previously reported that MCF-7 breast tumor cells are refractory to DNA damage induced apoptotic cell death (Magnet et al., in preparation; [10, 12, 28, 34, 41, 42]). It has been reported that idarubicin has the capacity to induce apoptosis in human lymphoma cells [36]. However, despite the approximately 70% reduction in cell numbers by exposure to idarubicin, four complementary analytical approaches (morphological analysis, static field gel electrophoresis, alkaline unwinding and in situ detection of DNA fragments) failed to provide evidence of apoptosis in MCF-7 cells exposed

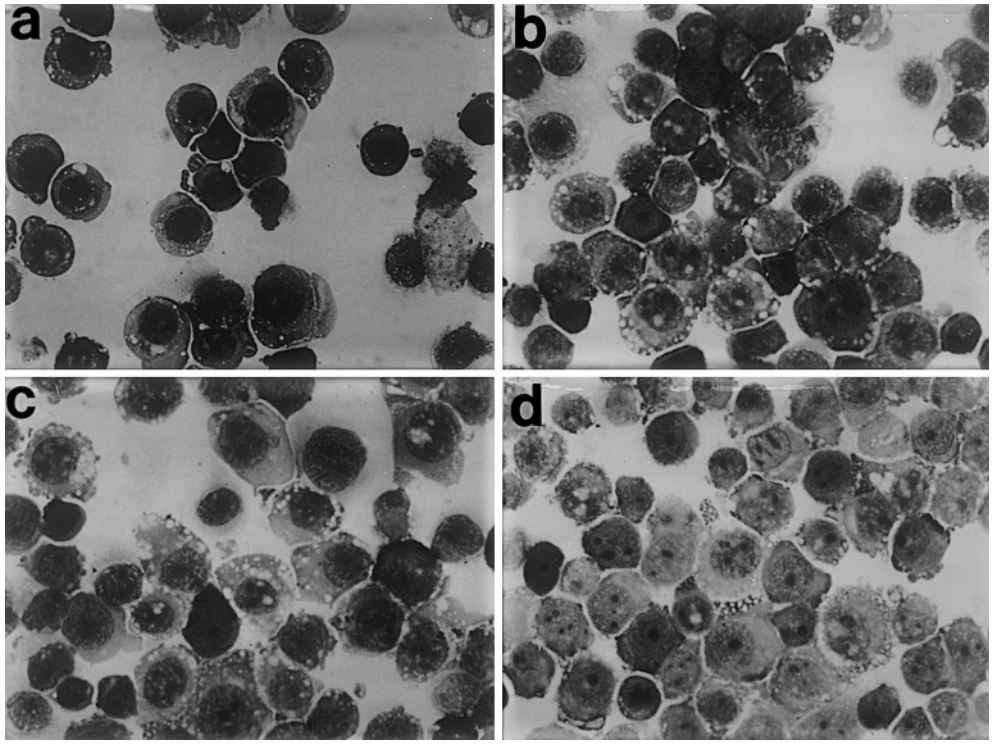


Fig. 6a–d Morphological effects of idarubicin in MCF-7 breast tumor cells as determined by light microscopy. Cells were exposed to 0.5 μ*M* idarubicin (**a** Control; **b** Idarubicin, 24 h; **c** idarubicin, 48 h; **d** idarubicin, 72 h)

to idarubicin. In view of the fact that MCF-7 cells apparently do have the capacity to undergo apoptotic cell death [22, 24, 26, 28, 30, 40], it appears possible that the refractoriness of these cells to apoptosis in the current and previous studies (Magnet et al., in preparation; [10, 12, 41]) may be related, in part, to suppression of c-*myc* expression, as c-*myc* is thought to contribute to p53 mediated apoptosis [19, 29].

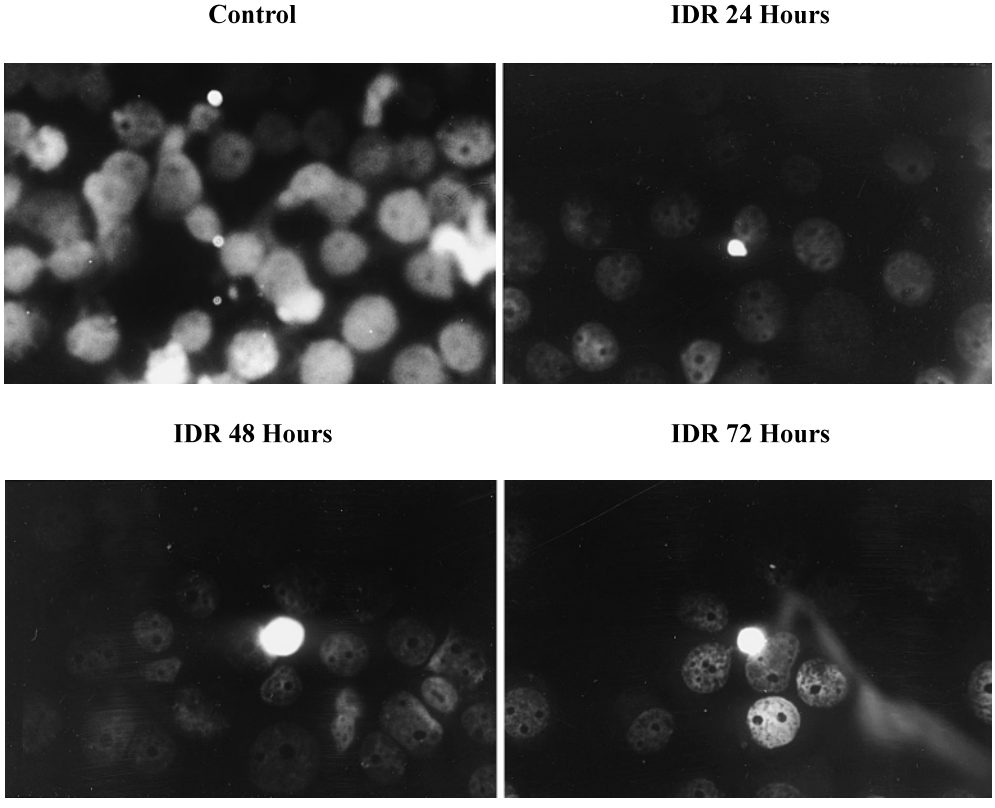


Fig. 7 TUNEL assay for the induction of DNA damage by idarubicin in MCF-7 breast tumor cells. MCF-7 cells were isolated on microscope slides at the indicated times after exposure to 0.5 μ*M* idarubicin and DNA fragmentation was assessed by fluorescent end-labelling

Inhibition of DNA biosynthesis and of c-*myc* expression by exposure to idarubicin

We as well as others have previously demonstrated a close correspondence between the effects of various topoisomerase II inhibitors on DNA synthesis and growth in MCF-7 cells [3, 11, 14, 37]. The present studies support and extend these prior observations to idarubicin.

As demonstrated for the topoisomerase II inhibitors, VM-26, m-AMSA and doxorubicin [3, 12, 14], and more recently for ionizing radiation [41], idarubicin produces a time- and concentration-dependent suppression of c-*myc* expression. Again, as reported for these other agents, the concentration-dependent suppression of c-*myc* expression by exposure to idarubicin corresponded closely with growth inhibition.

One possible explanation for the effects of idarubicin on c-*myc* expression is that idarubicin induces breaks within the c-*myc* gene that are not detected by conventional assays for DNA damage. We have previously reported that the topoisomerase II inhibitors, VM-26 and m-AMSA, induce damage within the locus of c-*myc* [3, 14]. Recently, Catapano et al. [6] have reported the existence of a topoisomerase II cleavage site in the first exon of c-*myc,* a finding consistent with earlier reports by other investigators [31, 33].

While the present studies in no way prove that altered c-*myc* expression is linked to the signal transduction pathway leading to growth arrest in MCF-7 cells, this hypothesis is strengthened by the correspondence between the early suppression of c-*myc* expression and the ultimate inhibition of growth for different topoisomerase II inhibitors as well as for ionizing radiation [3, 12, 14, 41]. One protein which could regulate c-*myc* expression is the transcription factor, E2F, which has binding sites in the promoter regions of a number of genes associated with DNA synthesis, including c-*myc* [8]. It is possible that alterations in c-*myc* expression are related to the fact that E2F is a component of the signal transduction pathway which responds to DNA damage through an increase in levels of the tumor suppressor protein p53, and the cyclin-dependent kinase inhibitor $p21^{waf1/cip1}$, and through dephosphorylation of the retinoblastoma tumor suppressor protein, Rb [8]. Studies are currently in progress to determine whether c-*myc* expression is regulated in response to DNA damage in other breast tumor cell lines and to identify the potential involvement of c-*myc* in the DNA damage response pathway.

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References

1. Belvedere G, Suarto A, Geroni C, Giuliani F, D'Incalci, MM (1989) Comparison of intracellular drug retention, DNA damage and cytotoxicity of derivatives of doxorubicin and daunorubicin in a human colon adenocarcinoma cell line (Lovo). Biochem Pharmacol 38: 3713
2. Berman E, McBride M (1992) Comparative cellular pharmacology of daunorubicin and idarubicin in human multidrug leukemia cells. Blood 79: 3267
3. Bunch RT, Povirk LF, Orr MS, Randolph JK, Fornari FA, Gewirtz DA (1994) Influence of m-AMSA on bulk and gene-specific DNA damage and c-myc expression in MCF-7 breast tumor cells. Biochem Pharmacol 47: 317
4. Capranico G, Soranzo C, Zunino F (1986) Single-strand DNA breaks induced by chromophore-modified anthracyclines in P388 leukemia cells. Cancer Res 46: 5499
5. Carella AM, Berman E, Maraone MP, Ganzina F (1990) Idarubicin in the treatment of acute leukemias: an overview of preclinical and clinical studies. Haematologia 75: 159
6. Catapano CV, Carbone GMR, Pisani F, Qiu J, Fernandes DJ (1997) Arrest of replication fork progression at sites of topoisomerase II-mediated DNA cleavage in human leukemic CEM cells incubated with VM-26. Biochemistry 36: 5739
7. Chirgwin JM, Pryzbyla HE, MacDonald RJ, Rutter WJ (1984) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18: 5294
8. Farnham PJ, Slansky JE, Kollmer R (1993) The role of E2F in the mammalian cell cycle. Biochim Biophys Acta 1155: 125
9. Ferrazzi E, Woynarowski JM, Arakali A, Brenner DE, Beerman TA (1991) DNA damage and cytotoxicity induced by metabolites of anthracycline antibiotics, doxorubicin and idarubicin. Cancer Comm 3: 173
10. Fornari FA, Jarvis WD, Grant S, Orr MS, Randolph JK, White FKH, Mumaw VR, Lovings ET, Freeman RH, Gewirtz DA (1994) Induction of differentiation and growth arrest associated with nascent (nonoligosomal) DNA fragmentation and reduced c-*myc* expression in MCF-7 human breast tumor cells after continuous exposure to a sublethal concentration of doxorubicin. Cell Growth Differ 5: 723
11. Fornari FA, Randolph JK, Yalowich JC, Ritke MK, Gewirtz DA (1994) Interference by doxorubicin with DNA unwinding in MCF-7 breast tumor cells. Mol Pharmacol 45: 649
12. Fornari FA, Jarvis WD, Orr MS, Randolph JK, Grant S, Gewirtz DA (1996) Growth arrest via down-regulation of c-*myc* expression in MCF-7 breast tumor cells after acute exposure to doxorubicin. Biochem Pharmacol 51: 931
13. Gavrieli Y, Sherman Y, Ben-Sasson SA (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Bio 119: 493
14. Gewirtz DA, Fornari FA, Orr M, Randolph JK, Povirk L, Bunch RT (1993) Dissociation between bulk damage to DNA and the antiproliferative activity of teniposide in the MCF-7 breast tumor cell line: evidence for induction of gene-specific damage and alterations in gene expression. Cancer Res 53: 3547
15. Goebel M (1993) Oral idarubicin – an anthracycline derivative with unique properties. Ann Hematol 66: 33
16. Goldenberg GJ, Wang H, Blair GW (1986) Resistance to adriamycin: relationship to cytotoxicity of drug uptake and DNA single- and double strand breakage in cloned lines of Adriamycin-sensitive and resistant P388 leukemia. Cancer Res 46: 2978
17. Grant S, Jarvis WD, Swerdlow PS, Turner AJ, Traylor RS, Wallace HJ, Lin PS, Pettit GR, Gewirtz DA (1992) Potentiation of the activity of 1-β-D-arabinofuranosylcytosine by the protein kinase C activator bryostatin-1 in HL-60 cells: association with enhanced fragmentation of mature genomic DNA. Cancer Res 52: 6270
18. Henderson IC, Canellos GP (1980) Cancer of the breast: the past decade. New Engl J Med 302: 78
19. Hermeking H, Eick D (1994) Mediation of c-Myc induced apoptosis by p53. Science 265: 2091
20. Jarvis DW, Kolesnick RW, Fornari FA, Traylor RS, Gewirtz DA, Grant S (1994) Activation of the sphingomyelin pathway initiates apoptosis in a variety of mammalian cell lines. Proc Natl Acad Sci 91: 73
21. Kanter PM, Schwartz HS (1982) A fluorescence assay for cellular DNA damage. Mol Pharmacol 22: 145
22. Kiguchi K, Glesne D, Chubb CH, Fujiki H, Huberman E (1994) Differential induction of apoptosis in human breast

cancer cells by okadaic acid and related inhibitors of protein phosphatases 1 and 2A. Cell Growth Differ 5: 995
23. Kohn KW, Ewig RA, Erickson L, Zwelling L (1981) Measurement of strand breaks and cross links by alkaline elution. In: Friedburg EC, Hanawalt PC (eds) DNA repair: a laboratory manual of research procedures, vol 1. Marcel Dekker, New York, pp 379–402
24. Kyprianou N, English HF, Davidson NE, Isaacs JT (1991) Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. Cancer Res 51: 162
25. Martoni A, Piana E, Guaraldi MM, Cilenti G, Fartris A, Saccani F, Becchi G, Pannuti F (1990) Comparative phase II study of idarubicin versus doxorubicin in advanced breast cancer. Oncology 47: 427
26. McClosky, DE, Casero RA, Woster PM, Davidson NE (1995) Induction of programmed cell death in human breast cancer cells by an asymmetrically alkylated polyamine analog. Cancer Res 55: 3233
27. Munger C, Ellis A, Woods K, Randolph J, Yanovich S, Gewirtz D (1988) Evidence for inhibition of growth related to compromised DNA synthesis in the interaction of daunorubicin with H-35 rat hepatoma. Cancer Res 48: 2404
28. Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, Sikorska M (1993) Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. EMBO J 12: 3679
29. Packham G, Cleveland JL (1995) c-Myc and apoptosis. Biochim Biophys Acta 1242: 11
30. Pagliacci MC, Tognellini R, Grignani F, Nicoletti I (1991) Inhibition of human breast cancer cell (MCF-7) growth in vitro by the somatostatin analog SMS 201-995: effects on cell cycle parameters and programmed cell death. Endocrinology 129: 2555
31. Pommier Y, Orr A, Kohn KW, Riou JF (1992) Differential effects of amsacrine and epipodophyllotoxins on topoisomerase II cleavage in human c-myc protooncogene Cancer Res 52: 3125
32. Pronzato P, Bertelli G, Amoroso D, Fennuci C, Gardin G, Guido T, Rosso P (1991) Oral chemotherapy with idarubicin plus cyclophosphamide in advanced breast cancer. Chemotherapy 37: 449
33. Riou JF, Lefevre D, Riou G (1989) Stimulation of the topoisomerase II induced DNA cleavage sites in the c-myc protooncogene by antitumor drugs is associated with gene expression. Biochemistry 28: 9104
34. Sakakura C, Sweeney EA, Shirahama T, Igarashi Y, Hakomori S, Nakatani H, Tsujimoto H, Imanishi T, Ohgaki M, Ohyama T, Yamazaki J, Hagiwara A, Yamaguchi T, Sawai K, Takahashi T (1996) Overexpression of bax sensitizes human breast cancer MCF-7 cells to radiation induced apoptosis. Int J Cancer 67: 101
35. Schott B, Robert J (1989) Comparative cytotoxicity, DNA synthesis inhibition and drug incorporation of eight anthracyclines in a model of doxorubicin-sensitive and -resistant rat glioblastoma cells. Biochem Pharmacol 38: 167
36. Smith PJ, Rackstraw C, Cotter F (1994) DNA fragmentation as a consequence of cell cycle traverse in doxorubicin and idarubicin-treated human lymphoma cells. Ann Hematol 69: S7
37. Supino R, Necco A, Dasdia T, Casazza AMM, DiMarco A (1977) Relationship between effects on nucleic acid synthesis in cell cultures and cytotoxicity of 4-demethoxy derivatives of daunorubicin and adriamycin. Cancer Res 37: 4523
38. Tewey KM, Rowe TC, Yang L, Halligan BD, Liu LF (1984) Adriamycin induced DNA damage mediated by mammalian DNA topoisomerase II. Science 226: 466
39. Thomas PS (1980) Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc Natl Acad Sci 77: 5201
40. Warri AM, Huovinen RL, Laine AM, Martikainen PM, Harkonen PL (1993) Apoptosis in toremifene-induced growth inhibition of human breast cancer cells in vivo and in vitro. J Natl Cancer Inst 85: 1412
41. Watson NC, Di YM, Orr MS, Fornari FA, Randolph JK, Magnet KJ, Jain PT, Gewirtz DA (1997) The influence of ionizing radiation on proliferation, c-*myc* expression and the induction of apoptotic cell death in two breast tumor cell lines differing in p53 status. Int J Radiat Biol 75: 547
42. Whitacre CM, Berger NA (1997) Factors affecting topotecan-induced programmed cell death: adhesion protects cells from apoptosis and impairs cleavage of poly (ADP-ribose) polymerase. Cancer Res 57: 2157
43. Woods KE, Ellis AL, Randolph JK, Gewirtz DA (1989) Enhanced sensitivity of the rat hepatoma cell to the daunorubicin analogue 4-demethoxydaunorubicin associated with induction of DNA damage. Cancer Res 49: 4846
44. Zwelling LA, Bales E, Altschuler E, Mayes J (1993) Circumvention of resistance to doxorubicin, but not by idarubicin in a human leukemia cell line containing an intercalator-resistant form of topoisomerase II: evidence for a non-topoisomerase II-mediated mechanism of doxorubicin toxicity. Biochem Pharmacol 45: 516.