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Induction of apoptosis in estrogen dependent and independent breast cancer cells by the marine terpenoid dehydrothyrsiferol

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Abstract

Breast cancer (BCA) represents the highest incidence of death in 35- to 60-year-old women. Above all, hormone unresponsive BCA is still associated with poorer prognosis than hormone receptor expressing malign, mammary tumors. There is a consistent need for effective compounds to treat especially the first variant of this disease. Therefore, we investigated the cytotoxic effects of the marine polyether triterpenoid dehydrothyrsiferol (DT) in four BCA cell lines. Annexin V labeling revealed higher rates of DT-induced apoptosis in hormone insensitive than in estrogen receptor expressing cells. Flow cytometric analysis of combined DNA fragmentation and total DNA labeling allowed us to ascribe apoptotic cells to their cell cycle stage. Although, high cell mortality was detected in mitogen dependent G_1-phase, time, concentration, and cell line dependent populations of apoptotic cells were also found to be of S-phase and G_2/M-phase origin. These results suggest that the induction of apoptosis by DT might be transduced through more than one effector pathway. Cell cycle distributions and 5-bromo-2′-deoxyuridine incorporation varied in a treatment dependent manner and differed from control experiments with colchicine and doxorubicin which exclude that DT functions as a mitosis inhibitor. In summary, we propose that DT might be an interesting candidate for an antitumor drug development regimen.

Keywords: Dehydrothyrsiferol (DT); Marine anticancer compound; Breast cancer; Apoptosis; Cell cycle distribution of apoptotic cells; BrdU incorporation

1. Introduction

Though there are geographical differences, BCA is still the leading cancer site in women worldwide [1]. Adjuvant hormone therapy is not indicated for patients with estrogen/progesterone receptor negative cancers [2,3], although this BCA is associated with poorer prognosis and shorter disease free and overall survival. In accordance with these facts, hormone receptor negative cell lines have been demonstrated to be more invasive and metastasis forming than their hormone-sensitive counterparts [4]. Therefore, the development of new, efficient chemotherapeutic agents, especially for the treatment of hormone unresponsive cancers is critical.

Apoptosis is an active process that regulates cell numbers and eliminates damaged cells. In contrast to necrosis, this tightly regulated and complex process exhibits some typical morphological changes, such as chromatin condensation, membrane blebbing, formation of apoptotic bodies, and in most cases, DNA fragmentation. Most of these changes are caused by a set of caspases, a subset of cysteine proteases, specifically activated in apoptotic cells. Although many of the key proteins in apoptosis have been

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Abbreviations: BCA, breast cancer; DT, dehydrothyrsiferol; FCS, fetal calf serum; Dox, doxorubicin; Col, colchicine; Ct, camptothecin; ER, estrogen receptor; BrdU, 5-bromo-2′-deoxyuridine; PI, propidium iodide; Tdt, terminal deoxynucleotidyltransferase; TUNEL, Tdt-mediated, dUTP nick end-labeling; Br-dUTP, bromodeoxyuridine triphosphate; FITC, fluorescein isothiocyanate; PS, phosphatidylserine; XTT, sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene sulfonic acid hydrate.

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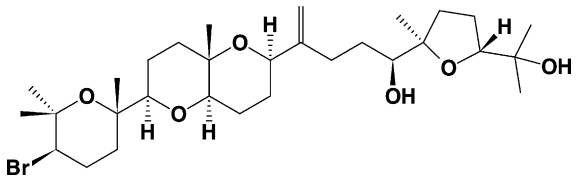

Fig. 1. Molecular structure of DT.

identified to date, a big part of the molecular mechanism of action or activation of these proteins has not been fully elucidated yet [5].

The marine terpenoid DT (Fig. 1) isolated from the indigenous Canarian red alga *Laurencia viridis spec. nov.* (Ceramiales, Rhodomelaceae) has been demonstrated to exhibit cytotoxic activity in a panel of cancer cell lines (P-388, A-549, MEL 28, and HT 29) [6]. Recently, a structurally related compound, thyrsiferol 23-acetate, has been shown to induce apoptosis under serum deprivation in T- and B-leukemia cells, but not in two myelocytic cell lines [7]. Thus, we were interested in characterizing the features of growth inhibition caused by DT in BCA cells and investigating whether DT induces apoptosis in this solid tumor model in a similar way.

To represent the two major classes of human mammary cancer cells, we chose for our study two estrogen receptor expressing (T47D and ZR-75-1) and two non-expressing (Hs578T and MDA-MB-231) BCA cell lines. In this report, we investigated the characteristics of the induction of apoptosis by DT in BCA cell lines under serum and growth factor maintaining conditions. Cell cycle changes were compared to those induced by the mitosis inhibitors Col and Dox. BrdU incorporation into DNA was analyzed in DT, as well as Col treated cells. Moreover, apoptotic cells were ascribed to their cell cycle stage.

2. Materials and methods

2.1. Drugs

DT (Fig. 1) was isolated by the Institute of Bio-Orgánica, Tenerife, Spain. It was prediluted in a 1:2 methanol/acetone solution, stored at −20° (1 mg/mL), and used within 5 months. Dox (hydroxydaunorubicin) was purchased from Pharmacia, stock diluted (0.8 mg/mL) in sterile water, and stored at −20°. Col (Sigma-Aldrich) was prediluted in DMSO (10 mg/mL) and stored at 4°. A 1 mM stock concentration of Ct aliquots (Sigma-Aldrich) in DMSO was stored at −20°. Influences of final concentrations of solution media in cell culture medium on experimental results were excluded by testing.

2.2. Cell culture

All cell lines were supplied from the American Type Culture Collection (ATCC). The estrogen receptor positive (ER^+) human BCA cell lines, T47D and ZR-75-1, were maintained in RPMI 1640 medium with 2 mM glutamine, 10% (v/v) FCS, 50 U/mL penicillin, and 50 μg/mL streptomycin. The estrogen receptor negative (ER^-) human BCA cell lines, Hs578T and MDA-MB-231, were cultured in DMEM high glucose medium (4.5 g/L glucose) and supplemented as above. T47D and Hs578T were, according to the ATCC instructions, additionally supplemented with 10 μg/mL bovine insulin. All mammary cell lines were controlled for their estrogen receptor status (not shown).

2.3. Cell proliferation and viability assay

The XTT tetrazolium assay (Roche Molecular Biochemicals) was carried out following the manufacturer's instructions and OD measured in an EL_X800NB ELISA reader (Bio-Tek Instruments) at a wavelength of 490 nm/ref. 630 nm. All tests were carried out in quadruplets. A direct modulating effect of the test compounds on the mitochondrial dehydrogenases, which metabolize the XTT to form a formazan dye, was excluded by comparing OD values of treated and untreated cells after 1 hr of incubation with the test drug. Controls with the vehicles used for stock solutions at experimental concentrations revealed no secondary influence.

2.4. IC_{50} values

Dose–response curves were calculated statistically by non-linear regression from the data measured by means of the XTT assay at different concentrations of compound (GraphPad Software). The IC_{50} concentrations were derived from these curves through the calculated absorbance at 50% growth inhibition.

2.5. BrdU incorporation

Cells were cultured in the presence vs. absence of DT or Col at 0.1 × IC_{50}, IC_{50}, and 2 × IC_{50} concentrations for 3 and 4 days (only DT), respectively. Using a BrdU ELISA system (Roche Molecular Biochemicals) as an alternative to the radioactive [^{3}H]thymidine incorporation assay, cells were reincubated with BrdU subsequently for 4 hr. Following the manufacturer's instructions, cells were then fixed and DNA denatured to allow additional access to the anti-BrdU peroxidase-labeled antibody. The following visible substrate oxidation was stopped by adding 1 M H_2SO_4. Absorbance was again measured in the EL_X800NB ELISA reader at a wavelength of 450 nm/ref. 630 nm.

2.6. Cell cycle analysis

Cells were trypsinized and adjusted to the appropriate cell number by dilution in PBS and then fixed in ice-cold 70% (v/v) ethanol until analysis (minimum 30 min). For DNA analysis cells were stained in a solution containing

1.17 mg/mL trisodium citrate, 250 μg/mL RNase A, 50 μg/mL PI, and 0.1% (v/v) Triton X-100 at 4° for 20 min. DNA histograms and dot plots were obtained using an FACSCalibur flow cytometer (Becton Dickinson). Twenty thousand events per sample were acquired. Doublets and cell debris were gated off in DNA dot plot view before calculation. Cell cycle distribution was calculated using ModFit software (Becton Dickinson).

2.7. Evaluation of apoptosis by annexin V staining

The topoisomerase I inhibitor, camptothecin, a known inducer of apoptosis [8], was used as a positive control at a final concentration of 0.2 μM with an incubation period of 3 days. DT effects were analyzed at 0.1 × IC_{50}, IC_{50}, and 2 × IC_{50} concentrations after 1, 2, and 3 days of incubation, respectively.

For evaluation of apoptosis, floating and carefully trypsinized cells were collected and washed in PBS. Staining with FITC-labeled annexin V and PI (Annexin V-FLUOS Staining Kit, Roche Molecular Biochemicals) was carried out following the manufacturer's instructions. Ten thousand events per sample were acquired and again analyzed on an FACSCalibur flow cytometer. Cell debris was excluded from calculation by gate setting in forward/sideward scatter view. Quadrant setting was fixed with untreated, single-stained controls and copied to dot plots of treated cells. Quadrant statistic calculations were carried out using CELLQuest software (Becton Dickinson).

2.8. Evaluation of apoptosis by Tdt-mediated nick end labeling

A variant of the TUNEL method was applied as a second method of confirming positive results of induction of apoptosis by DT. DNA labeling was carried out using the APO-BRDU Kit (Pharmingen, Becton Dickinson), following the manufacturer's instructions. Briefly, BrdUTP was added to 3′-hydroxyl ends of double- and single-stranded DNA break sites by the enzyme deoxynucleotidyltransferase in paraformaldehyde/ethanol fixed cells. The strand breaks were then identified by an FITC-labeled anti-BrdU monoclonal antibody. Total DNA was counterstained with PI. Twenty thousand events per sample were acquired and analyzed on the FACSCalibur flow cytometer using CELLQuest software. Doublets and cell debris were gated off in DNA dot plot view before calculating by means of quadrant statistic calculation.

3. Results

3.1. Cytotoxicity

IC_{50} values of DT, Dox, and Col in T47D, ZR-75-1, Hs578T, and MDA-MB-231 cells are given in Table 1.

Table 1
Cytotoxicity of DT, Dox, and Col in breast cancer cell lines

Cell line	DT	Dox	Col
ZR-75-1	9.4 ± 0.7	0.10 ± 0.02	0.055 ± 0.007
T47D	7.9 ± 0.6	0.03 ± 0.005	0.008 ± 0.002
Hs578T	11.1 ± 0.8	0.127 ± 0.02	0.002 ± 0.001
MDA-MB-231	14.8 ± 1.2	0.129 ± 0.03	0.004 ± 0.001

Cell viability was determined by the XTT assay after 48 hr of incubation with the tested drug. Data are given as means of IC_{50} values (μg/mL) ± SD from three experiments.

3.2. Results in ER^+ cells

3.2.1. BrdU incorporation

In general, IC_{50} and 2 × IC_{50} concentrations inhibited DNA synthesis to a high extent (Table 2). The 0.1 × IC_{50} concentrations of Col did not influence BrdU incorporation into DNA markedly, except for ZR-75-1 cells, which were treated at higher concentrations because of their previously shown Col resistance [9]. In contrary to Col, initial effects of DT were detected at 0.1 × IC_{50} concentrations in all cell lines after 3 days of incubation. Reduction of BrdU incorporation upon DT treatment was nearly continuous with rising concentrations and incubation periods (Table 2). T47D almost stopped replicating DNA only after 4 days (5.5% compared to control incorporation). ZR-75-1 still incorporated small amounts of BrdU, even after 4 days of incubation with the 2 × IC_{50} concentration of DT (approximately 12% of control incorporation).

Table 2
Effects of DT and Col on BrdU incorporation in BCA cell lines

	Days	Percent BrdU incorporation[a]		
		0.1 × IC_{50}	IC_{50}	2 × IC_{50}
DT treatment[b]				
ZR-75-1	3	90.2 ± 1.9	31.5 ± 1.8	23.8 ± 1.5
	4	79.4 ± 4.5	16.4 ± 0.7	11.4 ± 0.7
T47D	3	70.9 ± 4.0	23.9 ± 0.8	16.2 ± 0.5
	4	86.5 ± 4.2	24.7 ± 1.8	5.9 ± 0.2
Hs578T	3	68.1 ± 3.9	31.3 ± 0.9	14.5 ± 0.4
	4	59.3 ± 3.1	29.1 ± 1.0	8.5 ± 0.3
MDA-MB-231	3	96.2 ± 2.7	70.9 ± 1.8	5.8 ± 0.2
	4	91.3 ± 6.1	23.7 ± 1.9	4.8 ± 0.2
Col treatment[b]				
ZR-75-1		37.9 ± 1.0	18.2 ± 0.5	16.1 ± 0.2
T47D		98.6 ± 4.8	18.7 ± 0.5	15.7 ± 1.0
Hs578T		99.7 ± 5.3	37.8 ± 7.5	16.2 ± 0.5
MDA-MB-231		97.6 ± 1.7	31.2 ± 1.0	29.7 ± 1.0

[a] BrdU incorporation after 4 hr of additional incubation was detected by anti-BrdU peroxidase-labeled antibody. Results are shown as mean percent BrdU incorporation compared to untreated cells ± SEM of quadruplet samples.

[b] Cells were cultured for 3 and 4 days (DT) or 3 days only (Col) in a complete medium containing three distinct concentrations of DT and Col, respectively.

3.2.2. Cell cycle distributions

All BCA cell lines were assayed for their capacity to be synchronized by total serum withdrawal (data not shown). An increase in G_0/G_1-phase fractions from 8–18% was found in all cell lines. Cells that had been routinely cultured with insulin (T47D) exhibited a clear effect only when serum and insulin deprived.

3.2.2.1. Treatment with DT. Changes in distributions of cell populations in the various cell cycle phases after 1, 2, and 3 days of treatment with varying concentrations of DT are shown in Fig. 2. Briefly, $0.1 \times IC_{50}$ concentrations of DT provoked only mild effects in T47D cells whereas IC_{50} and $2 \times IC_{50}$ concentrations induced remarkable reductions in S-phase and increases in G_0/G_1-phase cells during all treatment periods. ZR-75-1 cells, although suffering small changes during $0.1 \times IC_{50}$ concentration exposure, seemed to be driven through cell cycle throughout the treatment. However, IC_{50} concentrations induced a clear accumulation in G_0/G_1 arrest phase with a concomitant reduction in S-phase. The $2 \times IC_{50}$ concentrations of DT induced a mixture of both patterns mentioned before.

3.2.2.2. Treatment with IC_{50} concentrations of Dox and Col. Controls with the standard chemotherapeutic compound Dox which, beside other effects, intercalates into the DNA and inhibits topoisomerase II, reduced G_0/G_1-phase and S-phase fractions markedly, and drove between 20% (ZR-75-1) and almost all of the cells (T47D) into G_2/M-phase after 3 days of incubation (Fig. 4). Col was used as an example for a mitosis inhibitor *via* microtubule disruption. Three days of exposure to Col induced decreases in G_0/G_1-phase and

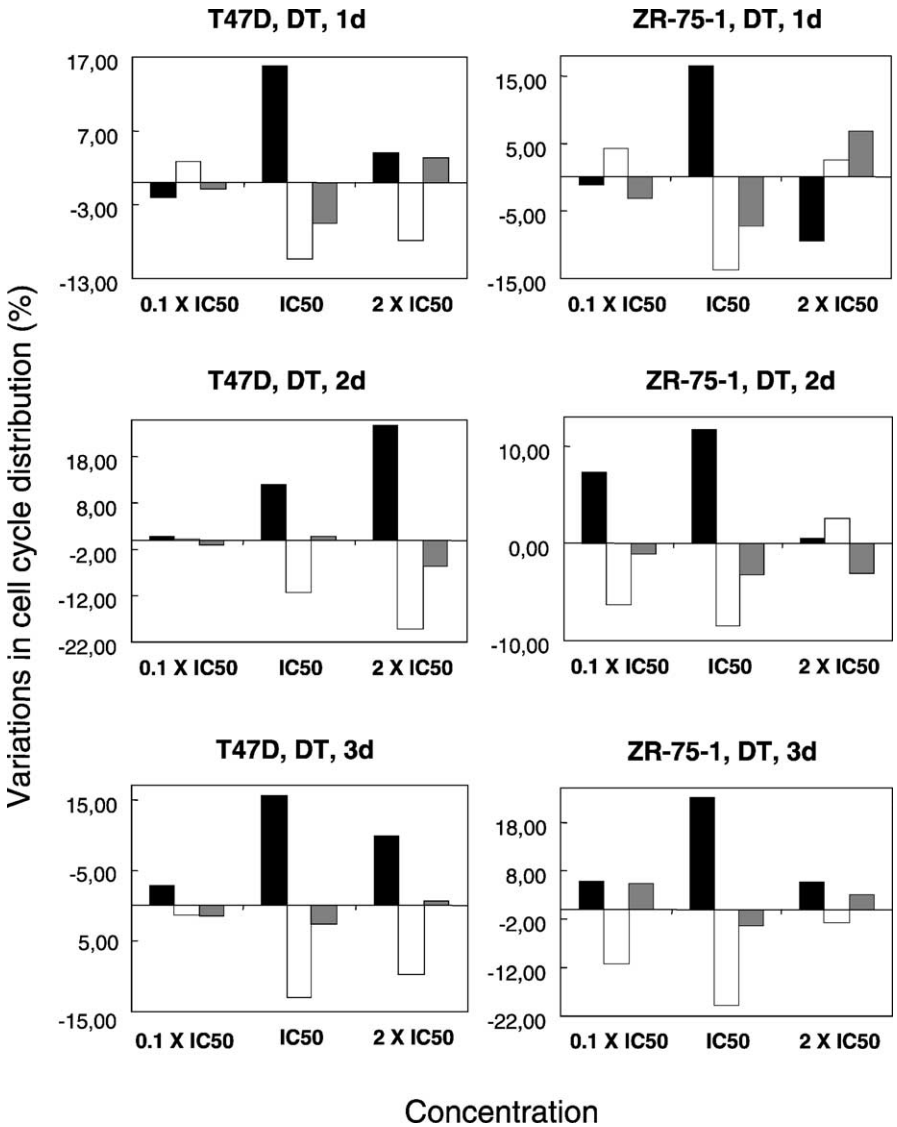

Fig. 2. Cell cycle distributions in ER^+ T47D and ZR-75-1 BCA cells upon treatment with DT. Changes in G_0/G_1- (■), S- (□), and G_2/M-phases (▩) following 1, 2, and 3 days of actuation of three distinct drug concentrations were analyzed by flow cytometry as described in Section 2. Variations in cell cycle distribution were expressed as percentages of treated cell populations minus percentages of untreated control cell populations.

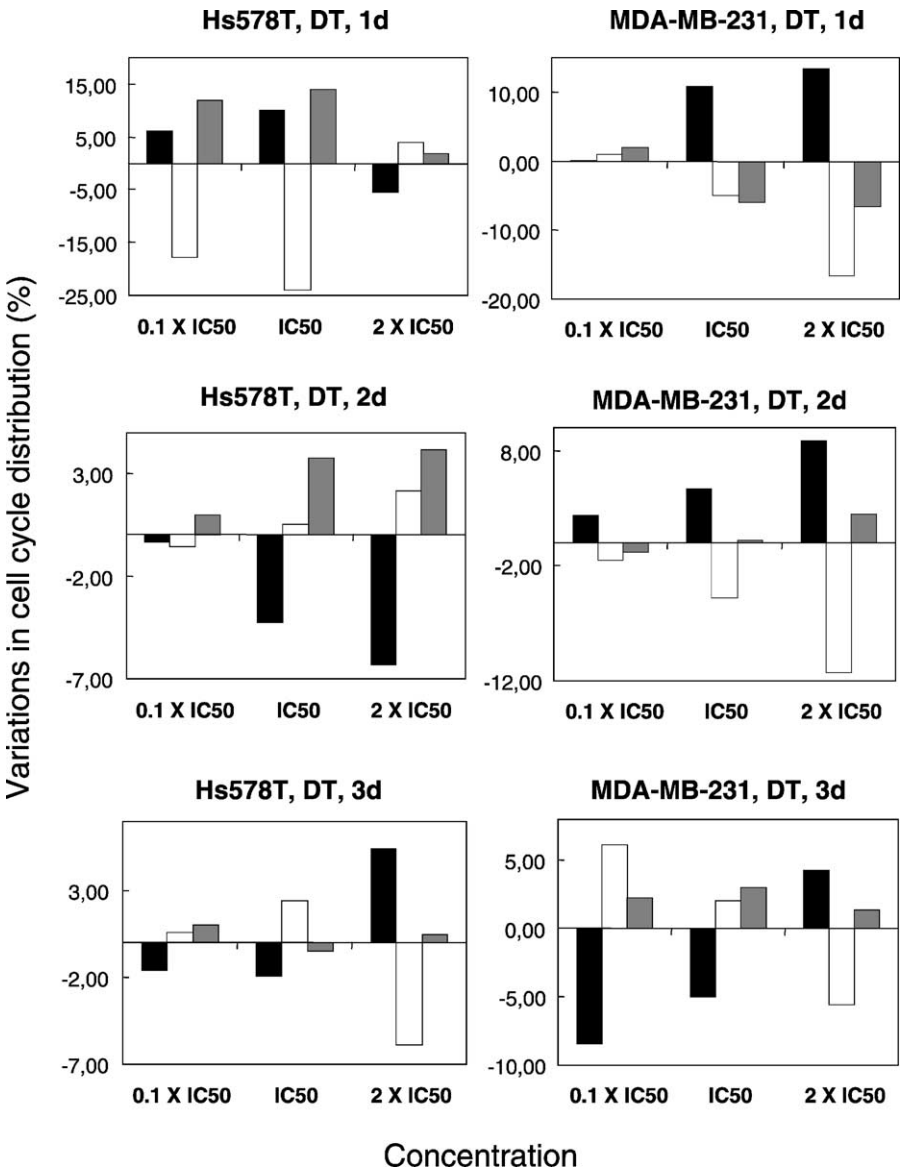

Fig. 3. Cell cycle distributions in ER$^-$ Hs578T and MDA-MB-231 BCA cells upon treatment with DT. Changes in G_0/G_1- (■), S- (□), and G_2/M-phases (▩) following 1, 2, and 3 days of actuation of three distinct drug concentrations were analyzed by flow cytometry as described in Section 2. Variations in cell cycle distribution were calculated as in Fig. 2.

concomitant increases either in S-phase and G_2/M-phase (T47D) or mainly in G_2/M-phase (ZR-75-1) (Fig. 4).

3.2.3. *Evaluation of apoptosis by determination of translocated phosphatidylserine (PS) at the cell surface*

Phagocytic marking by PS translocation from the inner membrane leaflet to the cell surface is a general feature of apoptotic cells [10,11]. Therefore, we decided to use annexin V, a specific ligand of PS, to determine the apoptotic fractions in the investigated cell types. Cells in necrosis, as well as late apoptosis, exhibit characteristic features, especially in regard to their membrane permeability. Therefore, cells were counterstained with PI which only passes cell membranes in the stage of necrosis or late apoptosis. Although the method does not allow distinguishing between these two stages of cell death (as annexin V would enter through membrane pores, as well as PI), we included the double positive cells in our considerations as possibly apoptotic cells.

3.2.3.1. Treatment with Ct. Three days of incubation with the topoisomerase I inhibitor and known inducer of apoptosis Ct [8] resulted in the following percentages of annexin V vs. double-stained cells: 21.7/39.5% in the ZR-75-1 and 15.8/29.2% in the T47D cell line (values of untreated control cells have been subtracted from these numbers).

3.2.3.2. Treatment with DT. DT effects were analyzed after 1, 2, and 3 days of incubation with 0.1, 1, and 2 × IC_{50} concentrations and are summarized in Table 3. T47D cells line exhibited only very slight, if any induction of apoptosis after 1 and 2 days of incubation with any concentration of DT.

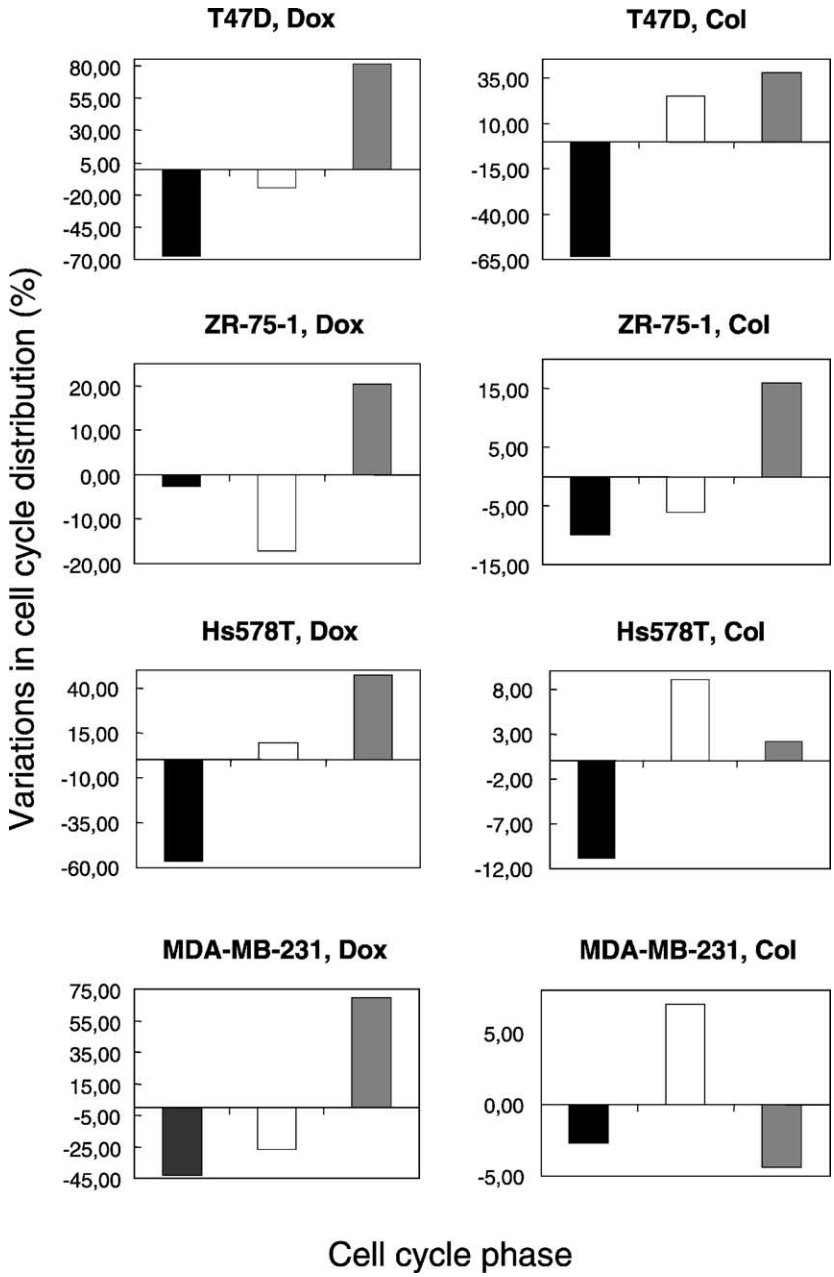

Fig. 4. Cell cycle distributions in BCA cell lines upon treatment with Dox and Col. FACS analysis was performed as described in Section 2. Changes in G_0/G_1- (■), S- (□), and G_2/M-phases (▩) following 3 days of actuation of Dox or Col at the cell line-specific IC_{50} concentration were analyzed by flow cytometry as described in Section 2. Variations in cell cycle distribution were calculated as in Fig. 2.

On day 3, however, we observed increases of annexin V^+, as well as double-stained cells (e.g. 13% annexin V^+ and 10% double stained at $2 \times IC_{50}$ concentration). ZR-75-1 generally exhibited a similar pattern as T47D on days 1 and 2. Nevertheless, pronounced concentration-specific increases in the double positive cell populations were detected on day 3 of treatment.

3.2.4. Evaluation of apoptosis by Tdt-mediated nick end labeling

In many cases, apoptosis goes along with caspase-3-mediated activation of caspase-activated DNase (CAD), the nuclease responsible for the famous DNA fragmentation into multiple integers of approximately 180 base pairs. To confirm induction of apoptosis involving this mechanism, we carried out a variant of the TUNEL method, incorporating Br-dUTP at cleaved strand break sites and detecting them by an FITC-labeled Br-dUTP antibody. The method permitted PI counterstaining. Thus, we were able to plot apoptotic cells against their cell cycle stage. All four BCA cell lines were analyzed after 3 days of exposure to DT at $0.1 \times IC_{50}$, IC_{50}, and $2 \times IC_{50}$ concentrations (Table 4).

In T47D, no cells upon $0.1 \times IC_{50}$ treatment, 20% of the cells treated with IC_{50} concentration, and 40% of $2 \times IC_{50}$ treated cells were determined as apoptotic. Fig. 5 shows that cells with strand breaks mainly derived from G_0/G_1-phase,

Table 3
Assessment of translocated PS in DT treated BCA cells

	Cell line[a]							
	ZR-75-1		T47D		Hs578T		MDA-MB 231	
	ann[b]	ann/Pi[c]	ann[b]	ann/Pi[c]	ann[b]	ann/PI[c]	ann[b]	ann/PI[c]
3 day treatment								
0.1 × IC_{50}	6.4	11.8	6.2	0.4	0	−2.0	2.0	0
IC_{50}	6.0	51.0	1.8	3.0	3.5	4.0	8.7	13.1
2 × IC_{50}	7.5	78.2	13.1	9.8	28.5	13.5	25.3	3.4
2 day treatment								
0.1 × IC_{50}	2.5	2.9	−0.7	−0.2	−4.6	−6.6	12.1	5.6
IC_{50}	4.1	−6.4	2.1	7.3	−2.1	−0.2	4.8	10.3
2 × IC_{50}	−4.9	0.7	−0.4	−5.2	12.7	9.6	31.2	−10.5
1 day treatment								
0.1 × IC_{50}	0.1	0.3	2.4	−2.7	2.7	9.7	−1.7	0.8
IC_{50}	1.9	1.3	5.1	2.3	−2.1	4.2	−0.6	0.8
2 × IC_{50}	2.1	0.9	3.4	5.7	6.3	9.5	5.7	4.8

[a] Cells were grown in a complete medium containing three distinct concentrations of DT for 1, 2, and 3 days.
[b] Percentages of cells with PS being translocated to the cell surface were determined by annexin V/FITC labeling (ann) and FACS analysis. Basic values of annexin V^+ untreated control cells were subtracted.
[c] PI counterstaining (ann/PI) revealed cells with pronounced membrane damage as occurs in late apoptotic, but also in necrotic cells. Basic values of double-stained untreated control cells were subtracted.

but also from G_2/M-phase. In the ZR-75-1 cell line, 8% of 0.1 × IC_{50} treated, 65% of IC_{50} treated cells, and 77 % of 2 × IC_{50} treated cells were identified to be in apoptosis after 3 days. Cell cycle stages of the apoptotic population varied more with treatment concentration, including S-phase cells (upon 2 × IC_{50} treatment). However, cells from G_0/G_1-phase again dominated the apoptotic fraction (Fig. 5).

3.3. Results in ER^- cells

3.3.1. BrdU incorporation

IC_{50}, as well as 2 × IC_{50} concentrations, of both DT as well as Col inhibited DNA synthesis to a high extent (Table 2), although BrdU incorporation in ER^- cells was less affected by IC_{50} concentrations than in their ER^+ counterparts. MDA-MB-231 cells exhibited an almost complete stoppage of BrdU incorporation at 2 × IC_{50} concentration of DT after 3 days of incubation (5.8%), while Hs578T ceased replicating DNA only after 4 days (8% compared to control BrdU incorporation). The 0.1 × IC_{50} concentrations of Col did not influence BrdU incorporation into DNA markedly. Nevertheless, initial effects of DT were measured at 0.1 × IC_{50} concentrations after 3 days of incubation. The pattern of decreased BrdU incorporation upon DT treatment was again almost continuous with rising concentrations and incubation periods (Table 2).

Table 4
Evaluation of apoptosis by TUNEL

Cell line[a]	Percent nick end-labeled cells[b]		
	DT (concentration)		
	0.1 × IC_{50}	IC_{50}	2 × IC_{50}
ZR-75-1	8.5	65.3	76.9
T47D	0.1	20.2	40.0
Hs578T	1.0	2.5	98.9
MDA-MB-231	2.3	10.5	98.7

[a] Cells were grown in a complete medium containing three distinct concentrations of DT for 3 days.
[b] Percentages of cells with DNA fragmentation (nick ends) were assessed and analyzed by flow cytometry. Basic values of untreated control cells were subtracted.

3.3.2. Cell cycle distributions

Hs578T which had been routinely cultured with insulin (as T47D) again exhibited a clear response to serum withdrawal only when deprived of both, serum and insulin.

3.3.2.1. Treatment with DT. Changes in distributions of cell populations in the various cell cycle phases after 1, 2, and 3 days of treatment with varying concentrations of DT are shown in Fig. 3. Hs578T was the only cell line which exhibited a dominant reduction in DNA synthesis at the beginning of the 0.1 × IC_{50} concentration treatment, but not on second and third days of exposure. However, the S-phase population was even more reduced after 1 day of actuation of DT at IC_{50} concentration. Longer incubations and/or a higher concentration did not influence cell cycle distributions in such a pronounced way.

A 0.1 × IC_{50} treatment of MDA-MB-231 cells induced a clear effect only on day 3. When incubated with IC_{50} concentrations, MDA-MB-231 accumulated in G_0/G_1-phase first, but ended up in a decrease in G_0/G_1-phase and a slight increase in S-phase and G_2/M-phase. However, the 2 × IC_{50} concentration induced a G_0/G_1-phase arrest on all three treatment days, thereby reducing the S-phase cell population remarkably.

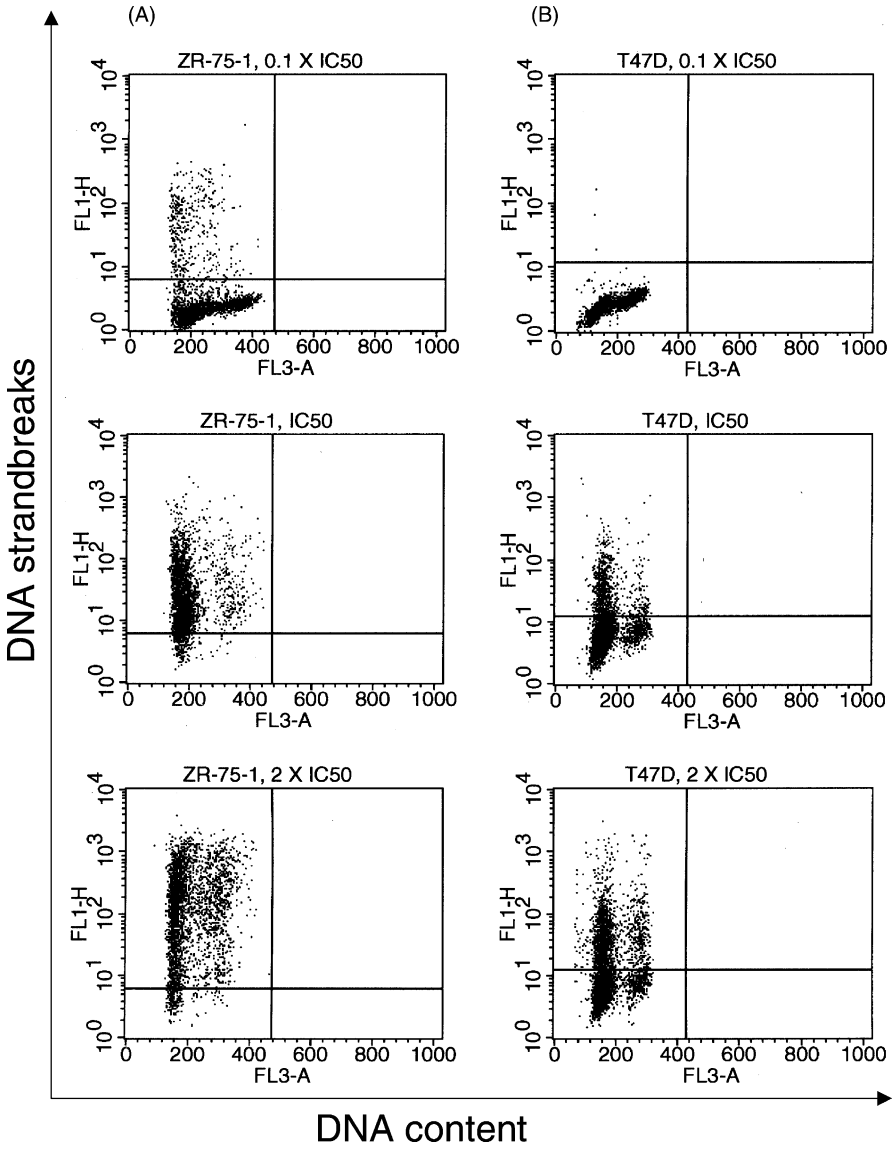

Fig. 5. Dot plot view of DNA content (*x*-axis) vs. quantity of DNA strand breaks (*y*-axis) of DT treated ZR-75-1 (A) and T47D (B) cells. Cell preparation and FACS analysis were carried out as described in Section 2. Quadrant settings were fixed with untreated control cells not crossing the lower, left quadrant (not shown). The left accumulation of cells in each plot represents cells with single DNA content in G_0/G_1-phase; the right one those with double DNA content in G_2/M-phase, both of them being connected by S-phase cells with intermediate DNA content.

3.3.2.2. Treatment with IC_{50} concentrations of Dox and Col. Control experiments with Dox reduced G_0/G_1-phase and S-phase fractions markedly and drove between 47% (Hs578T) and almost all of the cells (MDA-MB-231) into G_2/M-phase after 3 days of incubation (Fig. 4). Three days of exposure to the mitosis inhibitor Col resulted in small decreases in G_0/G_1-phase and concomitant increases mainly in S-phase in both ER^- cell lines (Fig. 4).

3.3.3. Evaluation of apoptosis by determination of translocated PS at the cell surface

3.3.3.1. Control treatment with Ct. Percentages of annexin V vs. double staining in the ER^- cell lines were as follows: 22.7/28.7% in Hs578T and 8.6/18.3% in MDA-MB 231 (basal values of annexin V^+ and double-stained untreated control cells have been subtracted from the data given above).

3.3.3.2. Treatment with DT. DT effects were again analyzed after 1, 2, and 3 days of incubation with 0.1, 1, and 2 × IC_{50} concentrations and are summarized in Table 3.

The apoptotic pattern of the two tested ER^- cell lines, Hs578T and MDA-MB-231, was found to be clearly distinct from the ER^+ ones. Hs578T did not exhibit induction of apoptosis when treated with 0.1 × IC_{50} and 1 × IC_{50} concentrations. But both, annexin V^+ as well as double positive cell populations, increased remarkably, time dependent upon 2 × IC_{50} concentration treatments.

MDA-MB-231 exhibited slight effects after 2 days of treatment with 0.1 × IC_{50} concentration and a time

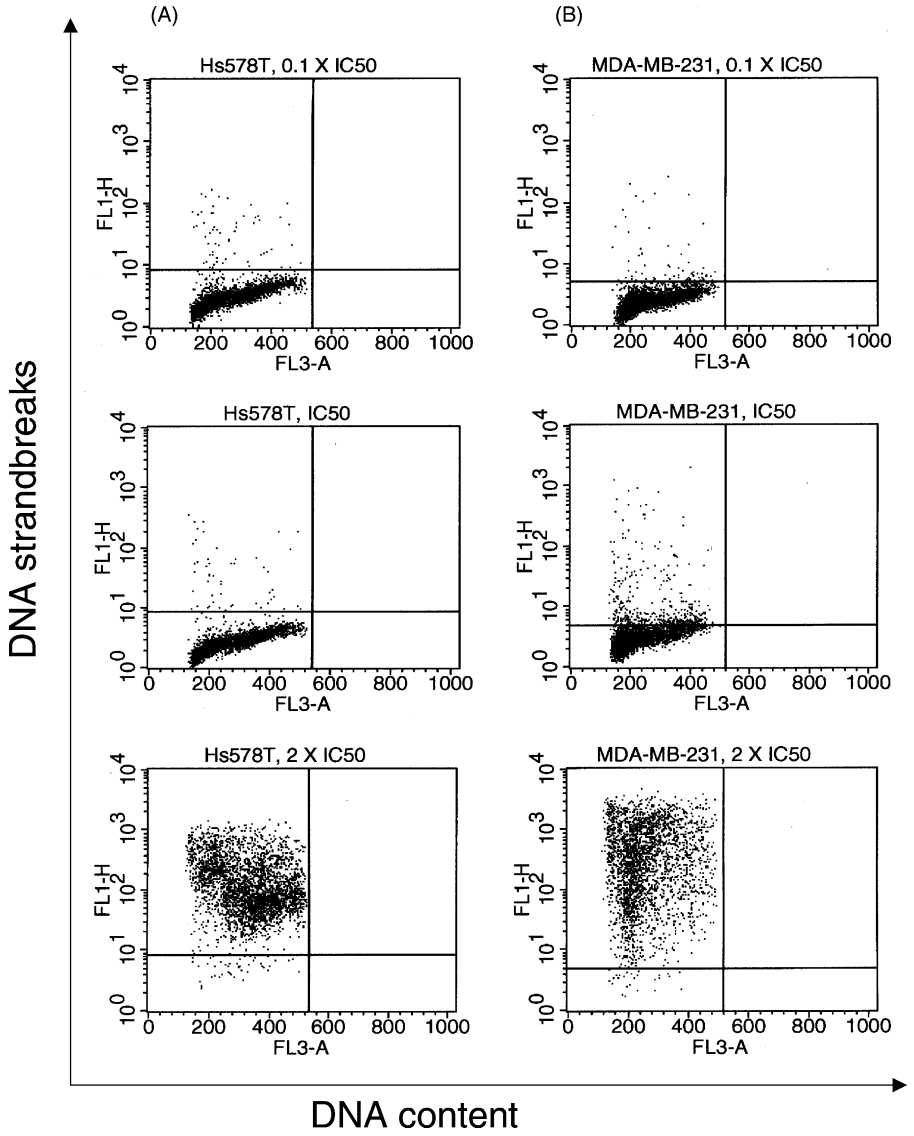

Fig. 6. Dot plot view of DNA content (*x*-axis) vs. quantity of DNA strand breaks (*y*-axis) of DT treated Hs578T (A) and MDA-MB-231 (B) cells. Cell preparation and FACS analysis were carried out as described in Section 2. Quadrant settings were fixed with untreated control cells not crossing the lower, left quadrant (not shown). The left accumulation of cells in each plot represents cells with single DNA content in G_0/G_1-phase; the right one those with double DNA content in G_2/M-phase, both of them being connected by S-phase cells with intermediate DNA content.

depending increase in annexin V and double positive cells during the IC_{50} treatment. The $2 \times IC_{50}$ concentration again proved to have the highest efficacy in driving the cells into apoptosis after 2 and 3 days of drug actuation.

3.3.4. Evaluation of apoptosis by Tdt-mediated nick end labeling

Results of the TUNEL analysis after 3 days of exposure to DT at 0.1, 1, and $2 \times IC_{50}$ concentrations are summarized in Table 4.

Although hardly any DNA fragmentation was induced with concentrations of up to IC_{50}, almost all Hs578T cells had shifted into apoptosis upon $2 \times IC_{50}$ exposure to DT for 3 days (99%). Still, the main bulk of the G_0/G_1-phase fraction exhibited higher fluorescence intensity with strand break staining than the main part of S-phase and G_2/M-phase cells, the latter ones not being clearly distinguishable.

A similar pattern was observed in MDA-MB-231, although a small apoptotic population was detectable in IC_{50} treated cells (10%). After 3 days of incubation with the $2 \times IC_{50}$ drug concentration, 99% of the cells demonstrated DNA cleavage, the G_0/G_1-phase-derived fraction again being the most dominant one (Fig. 6).

4. Discussion

The aim of the present study was to evaluate cytotoxic effects of the marine terpenoid DT in human BCA cell lines. We chose two ER^+ and two ER^- cell lines to represent both groups of patients' tumors.

Regarding the relatively homogenous IC_{50} values of DT in the investigated cell lines, one could have inferred that the metabolite induces a clearly defined growth inhibition pattern in BCA cells. To compare our data with well-described cytotoxic compounds, which are known to exhibit rather linear effects, we introduced Col and Dox into the study. They both interfere into the mitotic process, the first by tubulin disruption [12] and the latter by topoisomerase II inhibition [13].

4.1. ER^+ BCA cell lines

The capacity of Col to cause a mitotic arrest is known to be a very clear one [14]. Therefore, we compared BrdU incorporation into the DNA of DT treated vs. Col treated cells to find possible similarities. Only in ZR-75-1, which had proven to be highly Col resistant [9] and thus had to be treated with higher concentrations, an effect of $0.1 \times IC_{50}$ treatment was observed. In general, BrdU incorporation reached homogenous, but not minimal levels, upon Col treatment, although the ER^+ cells exhibited more sensitivity than the ER^- ones. The DT-induced inhibition pattern was different: In general, BrdU incorporation was reduced continuously with increasing both, DT concentration and exposure time in the ER^+ cells, even upon low concentration treatments clear effects were observed. Thus, there was no indication for a similar type of action through inhibition of mitosis as induced by Col.

Cell cycle studies also reflected the differences between DT and the two mitosis inhibitors, Dox and Col. Treatment with Dox caused ER^+ cell accumulations in the G_2/M-phase. Similar exposures to Col increased the G_2/M-phase population, although occasionally also induced increases in S-phase. The additional accumulations in S-phase may reflect the processing of the cells through the cell cycle modified by the cell line-specific doubling time or more likely, the concentration dependent effectiveness of the Col-mediated tubulin disruption [12]. However, comparing cell cycle patterns of Col and Dox with those achieved with DT, we were able to exclude the possibility that DT functions as a mitosis inhibitor.

Generally, changes in cell cycle distributions induced by DT seem to reflect a fluent process rather than a time dependent increasing accumulation of cells in an arrest stage. We observed a tendency of the ER^+ cell lines to accumulate in G_0/G_1-phase, but did not see similar cell cycle patterns throughout all treatment modalities. The ongoing variations with even sometimes reduction rather than accumulation in a specific phase suggested loss of cells by apoptosis.

In addition to first indications of apoptosis through observed morphological changes induced by DT, we detected concentration and time dependent external PS expressions in all investigated cell lines. In some cases, percentages of annexin V^+ cells were not very prominent (e.g. ZR-75-1 cells after 3 days of treatment). But annexin V^+/PI^+ cells made up a high percentage of these cell populations. As mentioned before, the method—although being very sensitive—does not permit differentiation between late apoptotic and necrotic cells. We presumed that, at least, part of the double positive cells were apoptotic.

To confirm this assumption, we also analyzed apoptotic DNA strand breaks after 3 days of DT exposure. Although the quantities of apoptotic cells were not completely consistent between the two methods applied, the quality of data was. Since the induction of apoptosis is a complex, multi-step event, we suggest that these differences are due to the distinct markers and differently time-regulated processes which were analyzed. Both methods can be assumed to be sensitive as they are based on detection of fluorescent signals. They also allow analysis of the complete cell population, including all weakly attached or floating cells, which might have been washed away with detection methods using microscopic slides. We propose that quantifying cells in apoptosis generally underlies method-related influences, and can only reveal relative, but not absolute differences in numbers.

However, clear induction of apoptosis was observed after 3 days of DT exposure with both test methods, although it was less complete in ER^+ cells than their ER^- counterparts.

4.2. ER^- BCA cell lines

With regard to BrdU incorporation, the more aggressive ER^- cell lines generally exhibited more resistance to Col treatment than the ER^+ ones, and once again did not decrease to minimal levels. Nevertheless, ER^- cells responded to DT treatment even at the lowest test concentration, and we were able to observe an almost complete stoppage of DNA synthesis at the highest test concentration.

Dox induced the same cell cycle effects as in the ER^+ lines (dominant accumulations in G_2/M), whereas Col treated cells tended to accumulate in S-phase. However, on the basis of both, BrdU incorporation and the cell cycle data, we were also able to exclude that DT functions as a mitosis inhibitor in ER^- BCA cells.

As Hs578T and MDA-MB-231 cells exhibited all sorts of altered cell cycle distributions, depending on DT treatment time and concentration, they reflected again the continuous processing of the cells through the cycle.

We observed PS positive cells from the first treatment day on and found markedly higher percentages of apoptotic cells compared to the ER^+ cell, which may explain the varying cell cycle patterns. Moreover, the ER^- cell lines proved to exhibit DNA strand breaks in almost all of the cells treated with the highest test concentration of DT. The flow cytometry plots of DNA fragmentation vs. DNA content give a qualitative impression of the cell cycle stage(s) out of which cells went into apoptosis. Generally, DT-induced apoptosis was observed in G_1 and G_2 arrest phase and—dose dependent—there were also some apoptotic cells with S-phase origin. With regard to the

high quantities of G_1-derived apoptotic cells, one might speculate about an onset of apoptosis in early G_1-phase—and, therefore, more time for ongoing DNA cleavage—than in the other cell cycle stages. In any case, further data would be necessary to support this highly hypothetic presumption.

Matsuzawa *et al*. [7] had demonstrated that thyrsiferol 23-acetate, a DT-related natural compound, induces apoptosis in T- and B-leukemic cell lines under low serum conditions. In their experimental setup, insulin revealed a protective effect with rising concentrations from 0.001 to 100 μg/mL. We carried out all our treatment experiments under normal growth conditions, i.e. in the presence of 10% FCS for all cell lines and 10 μg/mL insulin in the case of T47D and Hs578T, only omitting antibiotic treatment to avoid direct compound interactions. The fact that DT induces apoptosis in the presence of FCS and insulin suggests distinct and/or additional pathways compared to the effects of the structurally related thyrsiferol 23-acetate.

Moreover, our data suggest that there might be more than one mechanism involved in this process. The dissociation of the survivin–caspase-9 complex, e.g. is related to caspase-9 dependent apoptosis of cells traversing mitosis [15]. Sodium arsenite induces mitosis-mediated apoptosis, accompanied by delayed cyclin B degradation, in HeLa S3 cells which exit mitosis abnormally [16]. 5-Fluorodeoxyuridine arrests PC-3 prostate tumor cells in S-phase following apoptosis [17]. These examples demonstrate that apoptotic signaling, as well as apoptosis inducing anticancer compounds, may affect tumor cells in defined cell cycle stages. As we have demonstrated in this study, DT treated cells enter apoptosis from mitogen dependent (G_1 and transition to S), as well as independent (S and G_2), cell cycle phases. Thus, cell line-specific expression patterns alone cannot be made responsible for variable reactions following drug exposure (compare, e.g. [7]). Moreover, apoptosis was induced more efficiently and with distinct cell cycle-related patterns in the more aggressive ER^- cells than in their ER^+ counterparts.

Consequently, the effects of DT demonstrated in this study may be considered of interest for further elucidation of mechanisms of antineoplastic action and a resulting drug development regimen.

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