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Differential regulation of phosphatidylserine externalization and DNA fragmentation by caspases in anticancer drug-induced apoptosis of rat mammary adenocarcinoma MTLn3 cells

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Abstract

Caspase activation is a central event in the execution phase of apoptosis and is associated with phosphatidylserine (PS) externalization and DNA fragmentation. We investigated the role of caspase activity in anticancer drug-induced PS externalization and DNA fragmentation in MTLn3 cells. Caspase activation (DEVD-AMC cleavage) occurred in a time- and concentration-dependent manner after exposure to doxorubicin, in association with cleavage of poly(ADP) ribose polymerase and protein kinase C delta, two caspase-3 substrates. Caspase activation was closely followed by oligonucleosomal DNA fragmentation and PS externalization as determined by flow cytometric analysis. Similar observations were made for etoposide and cisplatin. Inhibition of caspases with zVAD-fmk inhibited almost completely doxorubicin-induced DNA fragmentation as well as proteolysis of protein kinase C delta. In contrast, PS externalization induced by doxorubicin was only partly affected by caspase inhibition. Flow cytometric cell sorting demonstrated that DNA fragmentation in the remaining PS positive cells after doxorubicin treatment in the presence of zVAD-fmk was fully blocked. In conclusion, these data indicate that while DNA fragmentation in anticancer drug-induced apoptosis of MTLn3 cells is fully dependent on caspase activity, PS externalization is controlled by both caspase-dependent and caspase-independent pathways.

Keywords: Cytostatics; Apoptosis; Phosphatidylserine; Cell cycle; Caspases; MTLn3 cells

1. Introduction

Apoptosis is an important mechanism by which anticancer drugs, including doxorubicin, etoposide and cisplatin, kill tumor cells [1,2]. Anticancer drug-induced apoptosis is tightly regulated by a variety of proteins. These include various Bcl-2 family members [3,4], apoptosis inhibitory proteins (IAPs) [5], p53 [6,7] and general growth-regulating and stress-activated signal transduction cascades [8,9]. The balance between the activity of these proteins determines the outcome of exposure to anticancer drugs: cell survival or death. Once the cellular commitment to apoptosis is made the family of cysteinyl aspartate specific proteinases, the caspases, are activated [10]. Thus, autocatalytic cleavage of either pro-caspase-8 or pro-caspase-9 through activation of the death receptor or mitochondrial pathway, respectively, results in cleavage and activation of pro-caspase-3 [11,12]. Active caspase-3 is crucial for the progression of many of the apoptotic events including nuclear fragmentation, cytoskeletal reorganization and membrane blebbing [10]. This is the direct result of (in)activation of various intracellular proteins due to proteolytic cleavage by caspase-3, and includes degradation of PARP[‡] [13], PKCδ [14], focal adhesion kinase [15] and fodrin [16].

Two fundamental events during apoptosis are oligonucleosomal DNA-fragmentation and externalization of phosphatidylserine (PS) at the outer leaflet of the plasma membrane [1,17]. PS-externalization is important for the phagocytosis of apoptotic cells by neighboring cells or macrophages [18–20]. Oligonucleosomal DNA fragmentation during apoptosis is a result of activation of caspase activated DNAse (CAD) [21,22]. Activation of CAD occurs after cleavage of the inhibitor of caspase activated DNAse

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Abbreviations: PS, phosphatidylserine; AV, annexin V; PI, propidium iodide; LDH, lactate dehydrogenase; zVAD-fmk, benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone; PKC, protein kinase C; PARP, poly-(ADP) ribose polymerase; AMC, 7-amino-4-methylcoumarin; alpha-MEM, alpha minimal essential medium; and FBS, fetal bovine serum.

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(ICAD) by caspase-3 [21]. Thus, MCF-7 breast tumor cells that lack functional caspase-3 are unable to fragment their DNA after induction of apoptosis by hydrogen peroxide; however, externalization of PS still takes place [23]. In FAS-L-induced apoptosis both DNA fragmentation and PS externalization are dependent on caspase activation [24]. Little is known about the role of caspases in the PS externalization and DNA fragmentation in tumor cells after exposure to anticancer drugs.

The rat mammary adenocarcinoma cell line MTLn3 is often used as a tool to study molecular mechanisms of metastasis formation [25,26] and responses to drug therapy both *in vitro* and *in vivo* [27,28]. These cells can be genetically modified *in vitro* and subsequently inoculated *in vivo* in syngeneic female Fisher 344 rats, allowing the study of molecular mechanisms of anticancer drug-induced apoptosis both *in vitro* and *in vivo*. We have used these cells to determine the role of caspases in anticancer drug-induced externalization of PS and oligonucleosomal fragmentation of DNA. The data indicate that both doxorubicin, etoposide, and cisplatin cause a time- and concentration-dependent externalization of PS and DNA fragmentation as determined by flow cytometric analysis. This is accompanied by the activation of caspase-3-like activity and caspase-3 substrate cleavage, which parallels the DNA fragmentation. DNA fragmentation caused by anticancer drugs is mediated fully by caspase-dependent pathways; in contrast, PS externalization is mediated by both caspase-dependent and -independent pathways.

2. Materials and methods

2.1. Chemicals

Alpha-modified minimal essential medium with ribonucleosides and deoxyribonucleosides (α-MEM) and penicillin/streptomycin were from Life Technologies. Fetal bovine serum (FBS) was from Bodinco. Doxorubicin, etoposide, cisplatinum (II) diamine dichloride (cisplatin), t-butyl hydroperoxide (t-BH), propidium iodide (PI), 7-amino-4-methylcoumarin (AMC), and RNAse A were from Sigma. Benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk), Acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin, acetyl-Val-Glu-Ile-Asp-7-amido-4-methylcoumarin, and acetyl-Tyr-Val-Ala-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC, Ac-VEID-AMC and Ac-YVAD-AMC, respectively) were from Bachem. Annexin V was from Boehringer Mannheim. Hoechst 33258 and the Alexa™ 488 protein labeling kit were from Molecular Probes. All other chemicals were of analytical grade.

2.2. Cell culture

MTLn3 rat mammary adenocarcinoma cells were provided by Dr. S. Jaken (Dept. of Pathology, University of Vermont, Burlington, VM, USA) and originally developed by Dr. D.R. Welch (Jake Gittlen Cancer Research Institute, The Pennsylvania State University College of Medicine, Hershey, PA, USA) and used between passages 46 and 56. They were cultured in α-MEM supplemented with 5% (v/v) FBS (complete medium). For experiments, cells were plated at a density of $4*10^3$ cells/cm^2 and grown for three days in complete medium supplemented with 50 U penicillin/L and 50 mg streptomycin/L (penicillin/streptomycin) in p60 or p90 plates (Corning) for flow cytometry, caspase activity assay, and immunoblotting. In the case of LDH release assays, cells were plated in 96-well plates at the same density. Cells were exposed to the anticancer drugs doxorubicin, etoposide, and cisplatin for 1 hr in Hanks' Balanced Salt Solution (137 mM NaCl, 5 mM KCl, 0.8 mM $MgSO_4 \cdot 7H_2O$, 0.4 mM $Na_2HPO_4 \cdot 2H_2O$, 0.4 mM KH_2PO_4, 1.3 mM $CaCl_2$, 4 mM $NaHCO_3$, 25 mM HEPES, 5 mM D-glucose; pH 7.4). After removal of the anticancer drugs, cells were recovered in α-MEM containing 1% (v/v) FBS and penicillin/streptomycin for the indicated periods. In some experiments, cells were recovered in α-MEM containing 1% (v/v) FBS, penicillin/streptomycin, and 100 μM zVAD-fmk. Exposure to t-butyl hydroperoxide was performed in αMEM containing 1% (v/v) FBS.

2.3. Determination of cell death

For *Annexin V/propidium iodide (AV/PI) staining*, cells were washed twice in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 2H_2O$, 1.4 mM KH_2PO_4; pH 7.4) containing 1 mM EDTA (PBS–EDTA) and subsequently trypsinized with 0.13 g/L of trypsin in PBS–EDTA. Medium, washes, and cells were combined, centrifuged (5 min, 200 *g*, 4°) and the pellet was washed once with PBS–EDTA. Cells were allowed to recover from trypsinization in complete medium (30 min, 37°). Cells were washed in PBS–EDTA and exposed phosphatidylserine (PS) was labeled (15 min, 0°) with Alexa488™-conjugated Annexin V in AV buffer (10 mM HEPES, 145 mM NaCl, 5 mM KCl, 1.0 mM $MgCl_2 \cdot 6H_2O$, 1.8 mM $CaCl_2 \cdot 2H_2O$; pH 7.4). Propidium iodide (2 μM) in AV buffer was added one minute prior to analysis by flow cytometry on a FACScalibur (Becton Dickinson).

For *cell cycle analysis*, trypsinized and floating cells were pooled and washed twice with PBS–EDTA and fixated in 70% (*v*/*v*) ice-cold ethanol (30 min, −20°). After two washes with PBS–EDTA, cells were incubated with PBS–EDTA containing 50 μg/mL of RNAse A and 7.5 μM PI (45 min, RT) and subsequently analysed by flow cytometry.

Caspase-3-like activity was determined as described by Jones *et al*. [29]. Briefly, cells were trypsinized as described for AV/PI, washed once in PBS–EDTA, and resuspended in lysis buffer (10 mM HEPES, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, pH 7.0). Cells were lysed by four cycles of freezing and thawing followed by centrifugation (30 min, 13,000 *g*, 4°). To 10 μg cell

lysate protein, 80 μL assay buffer was added (100 mM HEPES, 10% (w/v) sucrose, 0.1% (*v*/*v*) Nonidet P40, 10 mM dithiothreitol, 25 μM Ac-DEVD-AMC; pH 7.25) and the release of AMC was monitored (45 min, 37°) in a fluorescence plate reader (HTS 7000 Bio assay reader, Perkin Elmer). Free AMC was used as a standard and caspase activity was expressed as pmol AMC/min/mg protein.

Cell death was also monitored by the release of *lactate dehydrogenase (LDH)* into the medium as described [30]. Briefly, samples of the supernatant (30 μL) were taken at the indicated times. Total LDH activity was determined by lysing remaining cells with 0.1% (*w*/*v*) Triton X-100 in α-MEM containing 1% (*v*/*v*) FBS (15 min, RT). LDH activity assay was started by the addition of 200 μL assay buffer (200 mM Tris–HCl, 1 mM pyruvate, 0.4 mM NADH; pH 7.4) and the decrease in absorption at 340 nm was monitored continuously (5 min, RT) in the plate reader. LDH release into the medium was expressed as percentage of total LDH present in cell lysate (100%).

2.4. Cell sorting

In some experiments, AV^+/PI^- cells were sorted on a FACS Calibur in ice-cold PBS containing 1% (*v*/*v*) FBS. After centrifugation (10 min, 900 *g*, 4°) cells were fixed and stained in 3.7% (*v*/*v*) formaldehyde containing 2 μg/mL of Hoechst 33258 (15 min, RT). These cells were washed twice in PBS and dried on glass coverslips followed by mounting using Aqua PolyMount (Polysciences). Nuclear morphology was viewed using a fluorescence microscope. Total cell populations from the same treatment were used for comparison.

2.5. Immunoblotting

Attached cells were scraped in ice-cold PBS and pooled with medium containing floating cells. After centrifugation (5 min, 200 *g*, 4°) the pellet was resuspended in TSE (10 mM Tris–HCl, 250 mM sucrose, 1 mM EGTA, pH 7.4, containing 1 mM dithiothreitol, 10 μg/mL of leupeptin, 10 μg/mL of aprotinin, 1 mM sodium vanadate, 50 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride). The protein concentration in the supernatant was determined using the BioRad protein assay with IgG as a standard. Fifteen micrograms of total cellular protein was separated by SDS–PAGE and transferred to polyvinylidene fluoride membrane (Millipore). Blots were blocked with 5% (*w*/*v*) non-fat dry milk in TBS-T (0.5 M NaCl, 20 mM Tris–HCl, 0.05% *v*/*v* Tween 20; pH 7.4) and probed for PARP (monoclonal C2.10, Enzyme System Products), PKCδ (polyclonal δ14K, kindly provided by Dr. S. Jaken), caspase-9 (polyclonal, New England Biolabs), caspase-8 (kindly provided by Dr. J. Borst, Netherlands Cancer Institute, Amsterdam), caspase-3 or active caspase-3 (CSP3 and CM-1, respectively [31], kindly provided by Dr. A. Srinivasan, Idun Pharmaceuticals), followed by incubation with secondary antibody containing HRP and visualization with ECL reagent (Amersham Pharmacia Biotech).

2.6. Statistical analysis

Student's *t*-test was used to determine if there was a significant difference between two means ($P < 0.05$). When multiple means were compared, significance was determined by one-way analysis of variance (ANOVA; $P < 0.05$). For ANOVA analysis, letter designations are used to indicate significant differences. Means with a common letter designation are not different; those with a different letter designation are significantly different from all other means with different letter designations. For example, a mean designated as "a" is significantly different from a mean designated "b," but neither is different from a mean designated "a,b."

3. Results

3.1. Determination of doxorubicin, etoposide, and cisplatin-induced apoptosis in MTLn3 cells by DNA fragmentation

Apoptosis is characterized by nuclear condensation and oligonucleosomal DNA fragmentation. To determine the concentration–time course for the induction of apoptosis by various anticancer drugs, we first used flow cytometric analysis of the cell cycle. In control cells, the percentage of sub G_1/G_0 cells was 12 to 27% after 16 and 48 hr, respectively. This relatively high background level of apoptosis is most likely due to the fast growth rate of MTLn3 cells leading to high cell density which, in combination with low serum, results in apoptosis. Doxorubicin induced a concentration-dependent increase in sub G_1/G_0 cells at 16 hr after exposure, which was increased from 14% in control cells to 44% for 17 μM (Fig. 1A). The percentage sub G_1/G_0 cells increased further at 24 hr and was highest at 48 hr after exposure (68% at 17 μM). Etoposide (17–170 μM) induced a small increase in sub G_1/G_0 cells at 16 hr after exposure, which increased further after 24 (31% at 50 μM) and 48 hr (60% at 50 μM) (Fig. 1B). For cisplatin (30–100 μM), an increase in sub G_1/G_0 cells was not observed at 16 hr after exposure; at 24 hr the percentage of sub G_1/G_0 cells had increased to 34% (100 μM) and was also highest at 48 hr (69% at 100 μM, Fig. 1C). PI-stained nuclei from the flow cytometry samples were also counted under the microscope, which resulted in similar percentages of apoptotic nuclei (data not shown). In conclusion, doxorubicin, etoposide, and cisplatin caused apoptosis as determined by DNA fragmentation.

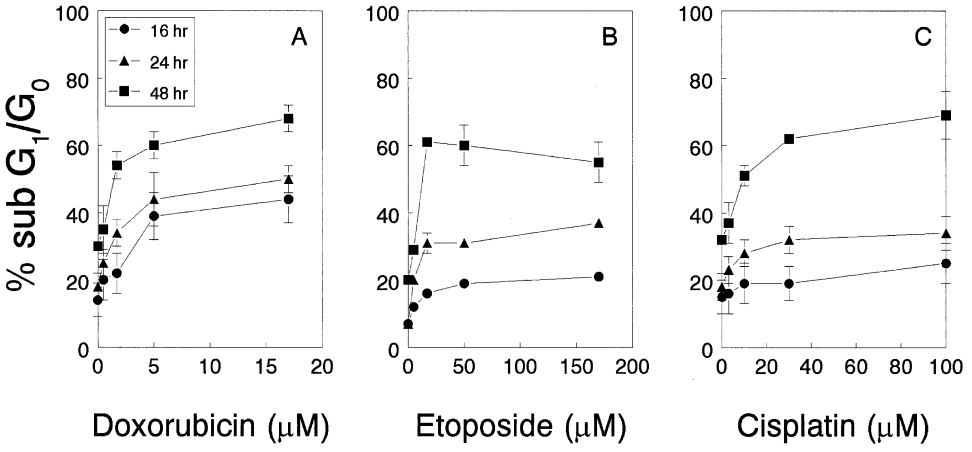

Fig. 1. The effect of doxorubicin, etoposide, and cisplatin on the loss of cellular DNA. MTLn3 cells were incubated with the indicated concentrations of doxorubicin (A), etoposide (B), or cisplatin (C) and the loss of cellular DNA (sub G_1/G_0) was determined by flow cytometry as described in Materials and methods. Data shown represent the means ± SEM of three independent experiments ($N = 3$).

3.2. PS externalization by doxorubicin, etoposide, and cisplatin

Next, we determined the externalization of PS to the outer leaflet of the plasma membrane, which is associated with apoptosis and important for phagocytosis of apoptotic cells [19]. PS externalization was determined by labeling cells with Alexa^{488}-labeled annexin V (AV) in combination with propidium iodide (PI) followed by flow cytometric analysis. AV/PI staining allows the discrimination between viable, apoptotic, and (secondary) necrotic cells. Doxorubicin induced AV^+/PI^--staining in up to 33% of cells at 16 hr after exposure to a concentration of 17 μM (Fig. 2A). The percentage of AV^+/PI^- cells was lower at 24 and 48 hr (28 and 23%, respectively), which coincided with a higher percentage of AV^+/PI^+ (i.e. necrotic) cells (up to 32% at 48 hr) (Fig. 2D). At concentrations below 17 μM, doxorubicin did not significantly induce AV^+/PI^- cells (Fig. 2A). Etoposide (17–170 μM) increased the percentage of AV^+/PI^- cells starting at 16 hr (16% at 50 μM) and reaching a maximum at 24 hr (22% at 50 μM); then it decreased again at 48 hr (12% at 50 μM, Fig. 2B). As the percentage of AV^+/PI^- cells decreased, the percentage of AV^+/PI^+ cells increased (Fig. 2E). Cisplatin (30–100 μM) only slightly increased the percentage of AV^+/PI^- cells at 24 and 48 hr (11 and 12% at 100 μM, respectively), which was not statistically significant (Fig. 2C). At 48 hr a drastic increase in AV^+/PI^+ cells was observed (Fig. 2F). Thus, although all the anticancer drugs caused an increase in sub G_1/G_0 cells, a different response is observed with regard to externalization of PS during apoptosis.

3.3. Role of primary necrosis of MTLn3 cells in DNA fragmentation

To check whether the increase in sub G_1/G_0 cells by anticancer drugs could be caused by primary necrosis, MTLn3 cells were treated with 200 μM t-butyl hydroperoxide (t-BH). Already after 8 hr, t-BH induced 54% AV^+/PI^+ cells (i.e. primary necrosis) and only 2% AV^+/PI^- cells (Table 1). In hypodiploidy analysis, these samples showed only 10% sub G_1/G_0 cells, indicating that this method does not generate a high percentage of false-positive cells.

3.4. Association of caspase-3-like activity with DNA fragmentation and PS externalization

Apoptosis is associated with activation of downstream or "executioner" caspases, including caspase-3 and -7. These two caspases preferentially cleave substrates at DEVD sequences. To determine the relationship between AV^+/PI^- staining, DNA fragmentation, and activation of caspases, we determined DEVDase activity in cell lysates of the same samples that were used for AV^+/PI^- staining and cell cycle analysis. Doxorubicin (17 μM) strongly induced DEVDase activity at 16 hr after exposure and the activity was maximal at 24 hr; it had slightly decreased again after 48 hr (Fig. 3C). Etoposide (170 μM) and cisplatin (100 μM) also increased DEVDase activity, resulting in maximal DEVDase activity at 48 hr (Fig. 3C). In control cells, no significant increase in caspase activity was observed. Next, we compared the caspase-3-like activity with caspase-6-like and caspase-1-like activities using Ac-DEVD-AMC, Ac-VEID-AMC, or Ac-YVAD-AMC as substrates, respectively. DEVDase activity was higher compared to VEIDase activity. No caspase-1-like YVADase activity could be detected (Table 2). To test whether caspase-3-like activity was functional in the apoptotic cells, we also determined whether typical caspase-3 substrates were cleaved. Indeed, the anticancer drug-induced increase in DEVDase activity correlated with cleavage of the major downstream caspase pro-caspase-3 (32 kDa) to its active (20 kDa) cleavage fragment. Both the time-course and the extent of cleavage followed the same pattern as DEVDase activity (Fig. 4). Moreover, activation

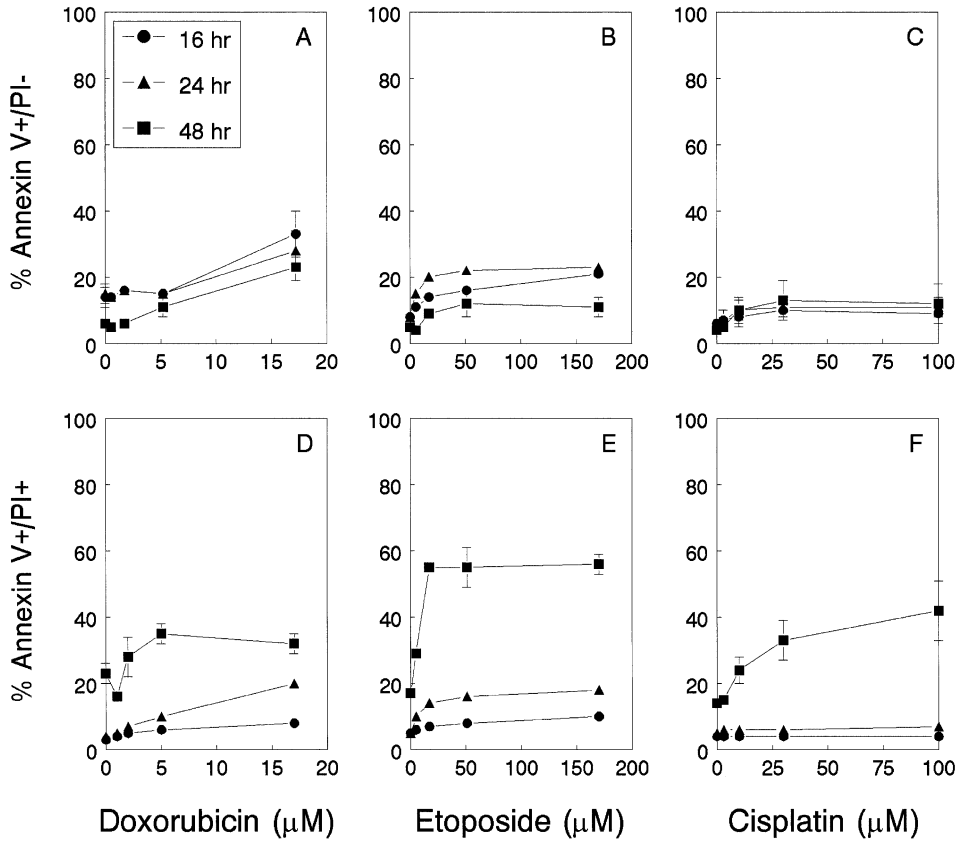

Fig. 2. PS externalization and loss of plasma membrane integrity are differentially induced by doxorubicin, etoposide, and cisplatin. MTLn3 cells were incubated with the indicated concentrations of doxorubicin (A, D), etoposide (B, E) or cisplatin (C, F), and the effect on PS exposure or apoptosis (Annexin V^+/PI^-, A–C) and on loss of plasma membrane integrity or necrosis (Annexin V^+/PI^+, D–F) was determined simultaneously by flow cytometry as described in Materials and methods. Data shown represent the means ± SEM of three independent experiments ($N = 3$).

of caspases was associated with time-dependent cleavage of typical caspase-3 substrates such as PKCδ and PARP. Doxorubicin induced complete degradation of PARP to the 85 kDa fragment after 16 hr. Incubation with etoposide and cisplatin resulted in cleavage of PARP that was only complete after 24 hr, which correlated to a later onset of DEVase activation (Fig. 3C) compared to doxorubicin.

Table 1
Primary necrosis does not induce an increase in $subG_1/G_0$ cells

	% AV^+/PI^- cells	% AV^+/PI^+ cells	% $subG_1/G_0$ cells
Control	1 ± 0	3 ± 1	3 ± 1
t-BH	2 ± 0	54 ± 18	10 ± 1

MTLn3 cells were treated with vehicle or 200 μM t-butyl hydroperoxide (t-BH) for 8 hr as described in Materials and methods. PS externalization (AV^+/PI^-), loss of plasma membrane integrity (AV^+/PI^+), and DNA fragmentation ($subG_1/G_0$) were determined in each sample by flow cytometry as described in Materials and methods. Data shown are means ± SEM from three independent experiments ($N = 3$).

3.5. Role of caspases in anticancer drug-induced DNA fragmentation and PS externalization

Next, we determined the causal relation between caspase activation on the one hand and DNA fragmentation and PS externalization on the other. For these and further experiments we used only doxorubicin, because it most strongly induced DNA fragmentation and PS externalization in MTLn3 cells. Since both DEVDase and VEIDase activity was increased in cells we used a general caspase inhibitor, zVAD-fmk, to inhibit all downstream effector caspases. ZVAD-fmk almost completely inhibited the doxorubicin-induced DNA fragmentation; there was no statistically significant difference in the percentage of cells with sub G_1/G_0 DNA content between control and doxorubicin/zVAD-fmk treatment (Fig. 5A). In contrast, the induction of AV^+/PI^- cells in the presence of zVAD-fmk was decreased by only 50% after exposure to doxorubicin (Fig. 5B); this was significantly different from control cells treated either with or without zVAD-fmk ($P < 0.001$). Importantly, this decrease in AV^+/PI^- cells did not result in an increase in AV^+/PI^+ cells (Fig. 5C), suggesting that ultimate cell death is caspase-independent. Moreover, despite the partial pro-

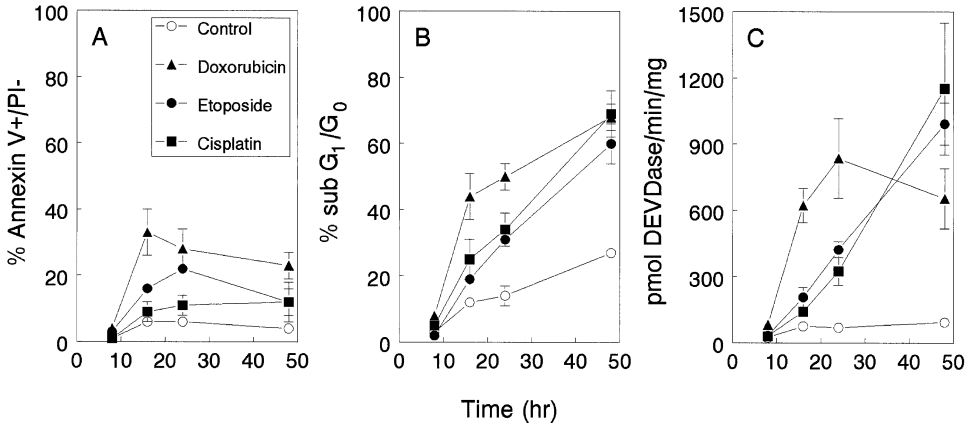

Fig. 3. Kinetics of Annexin V^+/PI^- staining, percentage of cells with sub G_1/G_0 DNA content, and caspase activity induced by anticancer drugs. MTLn3 cells were exposed to doxorubicin (17 μM), etoposide (50 μM), cisplatin (100 μM), or vehicle as described in Materials and methods. The percentage of Annexin V^+/PI^- cells (A) and of sub G_1/G_0 cells (B) and the caspase activity (C) were determined as described in Materials and methods. Data shown represent the means ± SEM of three independent experiments ($N = 3$).

tection against PS externalization, zVAD-fmk completely blocked the cleavage of pro-caspase-3 to its 20 kDa fragment (Fig. 6). In the presence of zVAD-fmk, doxorubicin-induced degradation of pro-caspase-9 was inhibited and the formation of the 36 and 40 kDa cleavage products of pro-caspase-8 was no longer observed. Cleavage of the caspase substrate PKCδ to the 40 kDa cleavage product was also inhibited by zVAD-fmk. In conclusion, both upstream and downstream caspases are inhibited by zVAD-fmk.

The above data indicate that regardless of the fact that zVAD-fmk blocked DNA degradation, AV^+/PI^- cells were still observed. Nuclear fragmentation is a hallmark for apoptosis and is dependent on caspase activity. Therefore, we checked whether zVAD-fmk blocked nuclear fragmentation even in AV^+/PI^- cells. To investigate this, AV^+/PI^- cells were sorted by flow cytometry and nuclei were stained with Hoechst 33258 and their morphology was analyzed by fluorescence microscopy. In the absence of zVAD-fmk, AV^+/PI^- cells from doxorubicin-treated cells had fragmented nuclei. In contrast, no fragmented nuclei were observed in AV^+/PI^- cells after treatment with doxorubicin in the presence of zVAD-fmk (Fig. 7).

Table 2
Anticancer drugs induce DEVDase and VEIDase but not YVADase activity

	DEVDase activity	VEIDase activity	YVADase activity
Control	69 ± 19	42 ± 7	nd[a]
Doxorubicin	836 ± 180	216 ± 82	nd[a]
Etoposide	422 ± 35	145 ± 12	0.5 ± 0.6
Cisplatin	324 ± 63	105 ± 9	nd[a]

MTLn3 cells were treated with vehicle, doxorubicin (17 μM), etoposide (50 μM), or cisplatin (100 μM) for 1 hr and allowed to recover for 24 hr as described in Materials and methods. The same samples were used to determine DEVDase, VEIDase, and YVADase activity (pmol/min/mg cell protein). Data shown are means ± SEM of three independent experiments ($N = 3$).

[a] nd: not detectable.

3.6. Sequential induction of apoptosis and necrosis by doxorubicin and differential involvement of caspases

Flow cytometric analysis of AV/PI-stained cells indicated that anticancer drugs also cause necrosis, i.e. AV^+/PI^+ cells, in particular at 48 hr (see Fig. 2). The increase in caspase activity in association with an increased percentage of cells with sub G_1/G_0 DNA content and PS externalization as well as cleavage of PKCδ occurred already after 16 hr (Figs. 3 and 4). This was well before an increase in PI^+ cells was observed. Therefore, loss of plasma membrane integrity as shown by PI permeability most likely occurs secondary to apoptosis, i.e. so-called secondary necrosis. To confirm this, we determined the extent of necrosis by analysing the release of the cytosolic enzyme lactate dehydrogenase (LDH) from cells after exposure to cytostatic drugs. Doxorubicin (17 μM) caused 13% LDH release at 24 hr, which further increased to 25% at 48 hr (Fig. 8). Similar findings were observed with etoposide and cisplatin. Finally, we determined the role of caspases in this necrosis. Although zVAD-fmk protected against doxorubicin-induced DNA fragmentation and caspase substrate cleavage, no protection was observed against release of LDH into the medium at 48 hr (Table 3), indicating that other cellular injury promotes the onset of necrosis. In conclusion, the data indicate that apoptosis is the primary cause of cell death that is later followed by plasma membrane permeabilization leading to secondary necrosis.

4. Discussion

Caspase activation is considered to be a central event in the execution phase of apoptosis [10]. Thus, many characteristics of apoptosis have been shown to depend on caspase activation, including DNA fragmentation, nuclear condensation and fragmentation, cleavage of caspase substrates, as

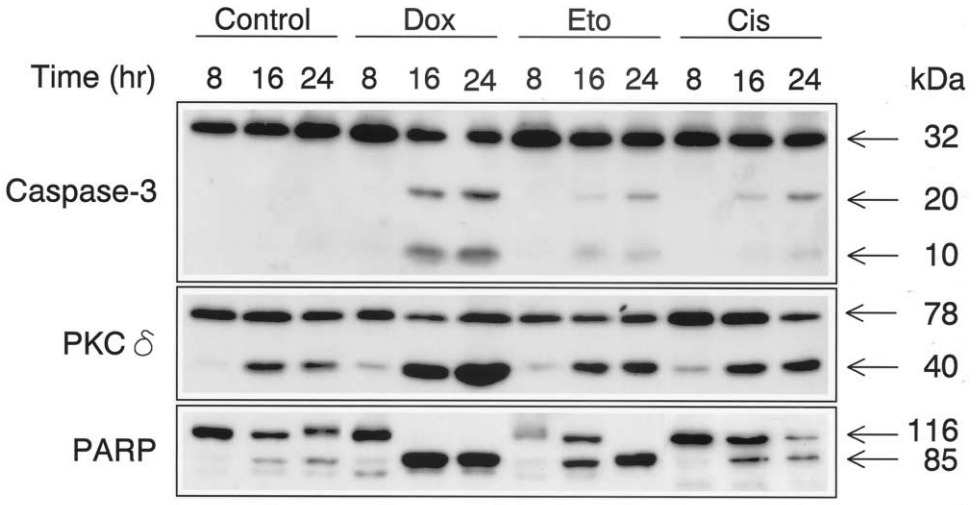

Fig. 4. Caspase-3, PKCδ, and PARP are cleaved during anticancer drug-induced apoptosis. MTLn3 cells were exposed to doxorubicin (Dox, 17 μM), etoposide (Eto, 50 μM), cisplatin (Cis, 100 μM), or vehicle as described in Materials and methods and allowed to recover for 8, 16, or 24 hr. Western blot analyses for caspase-3, PKCδ, and PARP were performed on total cell lysates. Indicated are the cleavage forms for caspase-3 (20 and 10 kDa), PKCδ (40 kDa), and PARP (85 kDa). Representative blots of three independent experiments are shown.

well as PS externalization [10,32]. Little is known about the relative importance of caspases in PS externalization and DNA fragmentation in anticancer drug-induced apoptosis of tumor cells.

The anticancer reagents we tested, i.e. doxorubicin, etoposide, and cisplatin, caused activation of caspase-3-like activity in MTLn3 cells which was associated with DNA fragmentation and PS externalization. Inhibition of caspase activity using the general caspase inhibitor zVAD-fmk prevented the DNA fragmentation. In contrast, PS externalization was only partially inhibited by zVAD-fmk; in the cells that still externalized PS in spite of the presence of zVAD-fmk, no nuclear changes could be observed. This indicates that these two apoptotic events are differentially regulated by caspases.

The requirement for caspases in PS externalization seems to be dependent on the nature of the apoptotic stimulus and cell type. Thus, a partial dependence of PS externalization on caspase activity has previously been observed in lymphocytes and erythroleukemia cells [33–35]. Caspase-3 does not seem to be involved in the induction of PS externalization, since MCF7 cells that lack caspase-3 can still externalize PS after apoptosis induction, although DNA fragmentation is absent [23]. In this case other downstream caspases, e.g. caspase-6, that can still be activated by caspase-9, may be responsible for the PS externalization. Caspase-independent signals may also trigger PS externalization. Thus, a rapid increase in intracellular free calcium may induce PS exposure [33,36]. Such PS exposure has been shown to be reversible if extracellular Ca^{2+} is chelated after PS externalization [33]. In addition, microinjection of apoptosis-inducing factor (AIF) has been shown to induce PS exposure even in the presence of zVAD-fmk [37].

Although inhibition of caspases blocked doxorubicin-

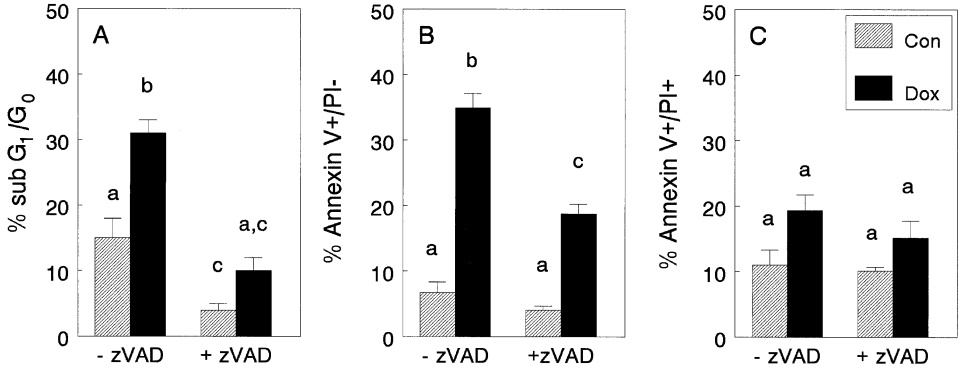

Fig. 5. zVAD-fmk largely blocks hypodiploidy whereas PS externalization is only partially blocked. Cells were exposed to vehicle or doxorubicin (Dox, 17 μM) for 1 hr in Hanks' balanced salt solution and subsequently recovered for 24 hr in the presence or absence of zVAD-fmk (100 μM). The percentage of sub G_1/G_0 cells (A), Annexin V^+/PI^- cells (B), and Annexin V^+/PI^+ cells (C) was determined as described in Figs. 1 and 2B. Data shown represent the means ± SEM of three independent experiments ($N = 3$) and the lower case letters indicate statistical significance as described in Materials and methods.

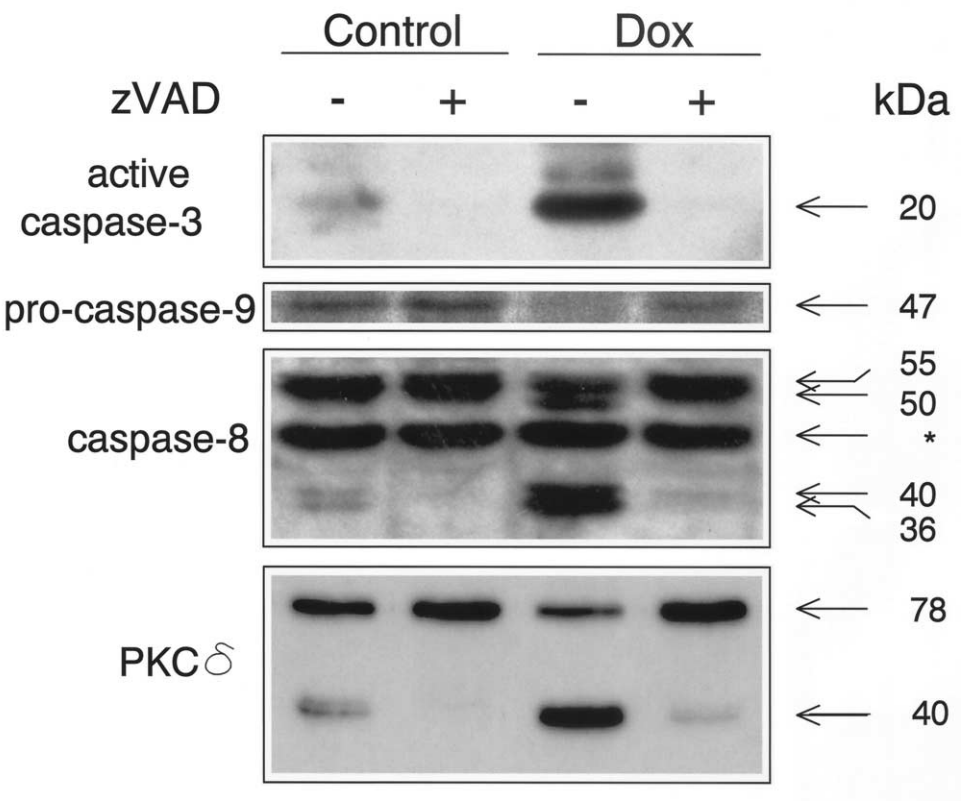

Fig. 6. Caspase substrate cleavage is blocked by zVAD-fmk. MTLn3 cells were treated with doxorubicin (17 μM) in the presence or absence of zVAD-fmk (100 μM) as described in Fig. 5. Western blot analysis for pro-caspase-9, caspase-8, active caspase-3, and PKCδ was performed on total cell lysates as described in Fig. 4. A representative blot of three independent experiments is shown. Note that pro-caspase-9 (47 kDa) and pro-caspase-8 (50 and 55 kDa) are not cleaved in the presence of zVAD-fmk and that the cleavage fragments of caspase-3 (20 kDa), caspase-8 (36 and 40 kDa), and PKCδ (40 kDa) also disappear in the presence of zVAD-fmk. The asterisk (*) indicates a non-specific band.

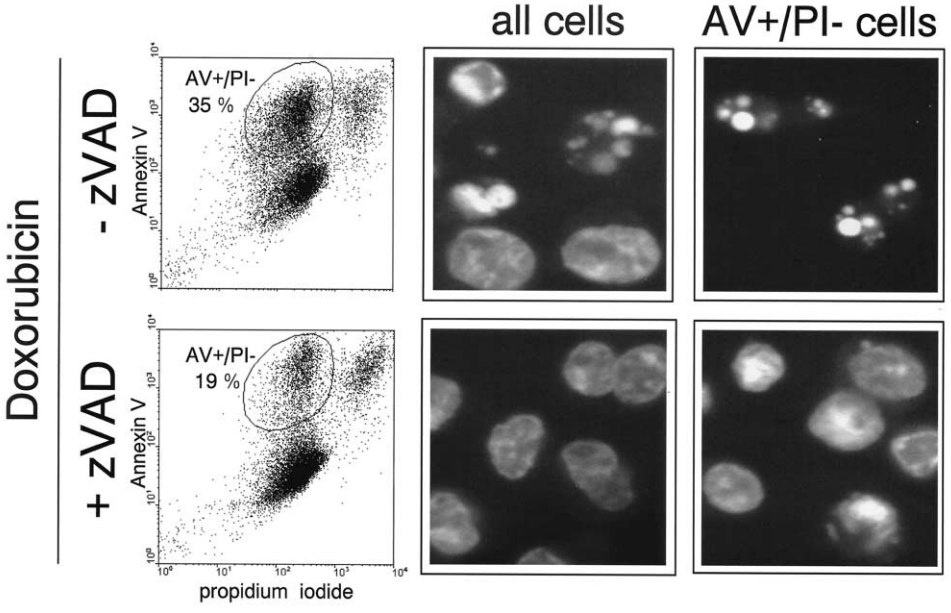

Fig. 7. zVAD-fmk blocks nuclear fragmentation in AV^+/PI^-. MTLn3 cells were treated with doxorubicin (17 μM) in the presence or absence of zVAD-fmk (100 μM) as described in Fig. 5. After 24 hr, samples were sorted by flow cytometry based on AV^+/PI^- characteristics (as indicated in the dotplots, left panel). After sorting, AV^+/PI^- cells were fixated with formaldehyde and nuclei were stained with Hoechst 33258 (right panel). Samples from the original population of cells were also collected and stained (middle panel). Representative pictures of three independent experiments are shown.

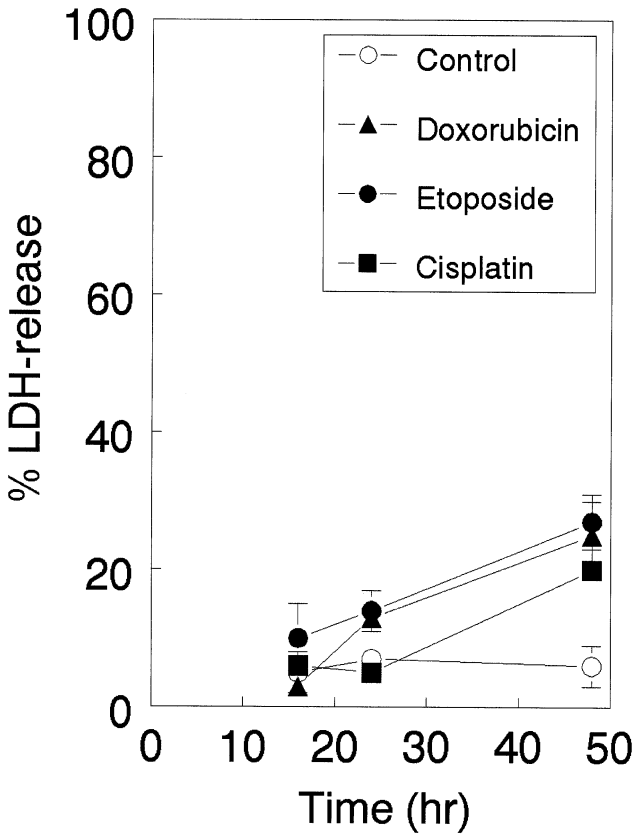

Fig. 8. Necrosis is a late event in anticancer drug-induced cytotoxicity. MTLn3 cells were exposed to doxorubicin (17 μM), etoposide (50 μM), cisplatin (100 μM), or vehicle as described in Materials and methods. Loss of plasma membrane integrity was determined by the release of the ubiquitous cytoplasmic protein lactate dehydrogenase (LDH) relative to the total amount present in the respective cells as described in Materials and Methods. Data shown represent the means ± SEM of three independent experiments ($N = 3$).

induced apoptosis, e.g. DNA fragmentation and caspase-3 substrate cleavage, ultimate cell death, i.e. necrosis, was independent of caspase activity (Fig. 5 and Table 3). Caspase-3 activation is often a downstream event resulting from mitochondrial perturbations including mitochondrial permeability transition. The permeability transition is modulated in part by the level and phosphorylation status of Bcl-2 family members such as Bcl-2 and Bax. Moreover, anticancer drugs can also directly affect mitochondrial functioning [38,39]. Thus, preliminary experiments indicate that doxorubicin also causes loss of the mitochondrial membrane potential, which seems to be independent of Bcl-2-mediated protection against apoptosis and caspase activation.[1] Such mitochondrial perturbations, eventually leading to ATP depletion, may explain the occurrence of necrosis even when caspases are inhibited.

The regulation of cell death induced by anticancer drugs is complex. It involves DNA and protein damage and oxidative stress [40,41], resulting in the up-regulation of transcription factors including c-jun [42], c-fos [43], gadd45 [44], gadd153 [45], and p53 [46], but also of proteins that are part of the apoptotic machinery, such as bax [47] and Fas/FasL [48]. The expression levels and posttranscriptional regulation of the latter proteins eventually determine commitment to cell death. Because of this complexity, the onset of commitment to apoptosis after exposure to anticancer drugs most likely varies between individual cells and may be spread over a long time period (up to 48 hr). To investigate the molecular mechanisms of cell death, it is important to be able to accurately monitor its kinetics. PS externalization is an early event in apoptosis and is therefore used as a marker for apoptosis by staining with annexin V [49]. However, the reliability of PS externalization as an apoptosis marker in time is very limited because apoptotic cells eventually lose their plasma membrane integrity, resulting in secondary necrosis, which is characterized by AV^+/PI^+ staining. Since primary necrosis also results in AV^+/PI^+ staining, the two processes are indistinguishable by Annexin V/PI staining. Consequently, only if the time-course of PS exposure is followed in detail and if the response of cells to apoptotic stimuli is rapid and involves a major part of the population (e.g. receptor-mediated apoptosis through the Fas pathway [50,51] or Natural Killer cell-induced cell killing [52]), changes in the percentage of apoptosis can be detected reliably by PS externalization. Yet, because of the variability of the cellular responses and the broad time span of the commitment to apoptosis in anticancer drug-induced apoptosis, it may not be possible to accurately follow anticancer drug-induced apoptosis in time using PS externalization. Indeed, our data show that the increase in AV^+/PI^- cells is transient: the maximal percentage of AV^+/PI^- cells is only approximately 30%, due to their loss of plasma membrane integrity, resulting in an increase in secondary necrotic AV^+/PI^+ cells. On the other hand, cells containing sub G_1/G_0 DNA content resulting from DNA fragmentation and degradation during apoptosis [53] will still be measured as such even if the cell membrane has become permeable due to secondary necrosis. Therefore, when using cell cycle analysis, all cells that are still *or have been* apoptotic until the moment of collection are

Table 3
Necrosis by anticancer drugs is not inhibited by zVAD

	% LDH release	
	−zVAD	+zVAD
Control	10 ± 5	10 ± 4
Doxorubicin	49 ± 8	44 ± 6

MTLn3 cells were treated with vehicle or doxorubicin (17 μM) as described in Materials and methods. Loss of plasma membrane integrity was determined at 48 hr after exposure by the release of LDH relative to the total amount present in the respective cells as described in Materials and methods. Data shown are means ± SEM from three independent experiments ($N = 3$).

[1]Huigsloot M, Van de Water B, manuscript in preparation.

cumulatively measured. As expected, this is also the case for apoptosis induced by anticancer drugs in MTLn3 cells (Fig. 3B). Despite the fact that primary necrosis does not appear to induce hypodiploidy (Table 1), it is recommendable to always measure necrosis (e.g. by LDH release or PI staining) to exclude an eventual necrotic component in the apoptotic population.

In conclusion, our data indicate that there is a differential role for caspases in the regulation of DNA fragmentation and PS externalization in anticancer drug-induced apoptosis. In addition, our quantitative evaluation of the time- and concentration-dependent induction of apoptosis as measured by caspase activity, cell cycle analysis, and PS externalization suggests that cell cycle analysis is a more optimal method to follow the kinetics of anticancer drug-induced apoptosis *in vitro*; caspase activity determination is a critical complementation to confirm apoptosis.

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