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APOPTOTIC CELL DEATH INDUCED BY DIFERENT TRIGGERING AGENTS MAY FOLLOW A COMMON ENZYMATIC PATHWAY

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Abstract

Molt-4 human leukemia cells were triggered to undergo apoptosis by various agents with different mechanisms of action. Staurosporine [a protein kinase C (PKC) inhibitor], camptothecin (a topoisomerase I blocking drug), and tiazofurin [an inhibitor of inosine 5'-phosphate dehydrogenase (IMPDH)], were used.

Ultrastructural analysis showed morphological changes characteristic for apoptosis that were very similar for all three agents. Nevertheless, DNA oligonucleosomic fragmentation was not detectable by agarose gel electrophoresis. However, a genomic DNA cleavage appeared after pulse-field gel electrophoresis (PFGE) in cells treated with these agents for 24 h. Furthermore, *in situ* nick translation (NT) showed a finely spotted nuclear labelling in all staurosporine-treated cells and a compact fluorescence after camptothecin incubation. In tiazofurin-treated cells an intermediate pattern was found. Therefore, apoptotis inducing agents with different mechanisms of action, induced the formation of large genomic DNA fragments and very similar ultrastructural changes. This could mean that these phenomena follow a pathway that is common to the three apoptosis-triggering agents, despite their different mode of action.

Key words: Apoptosis, Molt-4 cells, ultrastructure, DNA electrophoresis, nick translation.

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Introduction

Apoptosis is a physiologic phenomenon of cell death involved in the regulation of embryonic organ development and cell proliferation in normal and neoplastic tissues (Wyllie *et al*., 1980; Ellis *et al*., 1991; Kouri *et al*., 1992; Barr *et al*., 1994; Schwartz, 1995). Apoptosis appears in different cell types. It can also be experimentally induced in various cell types, and is accompanied by generally comparable structural changes (Falcieri *et al*., 1994c).

The molecular events underlying the apoptotic behaviour are mostly correlated to the activation of Ca^{2+}/Mg^{2+} dependent endonucleases, causing DNA cleavage in nucleosomic or oligonucleosomic fragments (Arends *et al*., 1990; Peitsch *et al*., 1993a,b). This pattern of DNA fragmentation, clearly identifiable by DNA agarose gel electrophoresis (Boe *et al*., 1991), has long been considered a hallmark of apoptosis.

Recently, a number of cell types has been described in which, despite the presence of other apoptotic signs, DNA oligonucleosomic fragmentation does not take place (Collins *et al*., 1992; Falcieri *et al*., 1993; Fady *et al*., 1994). Larger DNA fragments, prior to or in the absence of internucleosomic cleavage, were reported (Oberhammer *et al*., 1993a,b; Walker *et al*., 1995; Falcieri *et al*., 1996; Weaver *et al*, 1996). Apoptosis thus can proceed via different pathways and it is therefore advantageous to use a multiple technical approach for the study of this process.

In the present study, apoptosis was induced in human leukemia Molt-4 cells, and studied by different techniques. Three different agents, staurosporine, camptothecin and tiazofurin were used and compared. The role of cell metabolism and mechanism triggering the start and progression of apoptosis is discussed.

Materials and Methods

Cell culture and drug treatment

Molt-4 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and were maintained in RPMI1640 medium supplemented with 10% fetal

calf serum. All experiments were performed on cells during the exponential growth phase at 37°C.

Apoptosis was induced by 15 μM staurosporine (Boehringer, Mannheim, Germany) and 0.15 μM camptothecin (Sigma, U.K.) for 6, 18, 24 hours, and 10 μM tiazofurin (NSC 286193, kindly provided by Prof. G. Weber) for 24, 48, 72, 96 hours (Tricot *et al*., 1990).

Electron microscopy

Treated and control cells were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer and processed as previously described (Falcieri *et al*., 1994a).

Conventional agarose gel electrophoresis

DNA was routinely extracted (Boe *et al*., 1991), 4-5 μg were loaded on each lane and were run on 1.8% agarose gel for 120 minutes at 80 V and stained with 0.5 μg/ml ethidium bromide (Bissonnette *et al*., 1992).

Pulsed-field gel electrophoresis

Cell pellets, obtained from 5 x 10^7 cells/ml embedded in 1% low melting agarose, were incubated for 48 hours at 50°C in a lysis buffer containing 1% sarkosylate, 25 mM ethylenediaminetetraacetic acid (EDTA), 50 μg/ml proteinase K. The pellets were washed with 0.05 M EDTA and stored at 4°C. Runs of standard size DNA and Molt-4 cell DNA (after different treatments of the cells) was performed at 6 V/cm voltage, 14°C, 20/24 hour run times, a switch time from 45 to 90 seconds, 120° angle and linear ramping. The gel was finally stained with 5 μg/ml ethidium bromide for 30 minutes (Sambrook *et al*., 1989; Oberhammer *et al*., 1993 a,b; Walker *et al*., 1995, Falcieri *et al*., 1996).

***In situ* nick translation**

Cells, fixed in 3:1 methanol:acetic acid were layered on slides. Samples were then incubated for 45 minutes at room temperature in a solution containing 2 I.U. of endonuclease-free DNA polymerase I and 10 μM each of dATP, dCTP, dGTP and digoxigenin-dUTP in 50 mM TRIS-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol (Sigma, Italy). The slides were then washed for 5 minutes in 5% trichloroacetic acid at 4°C to remove the unincorporated triphosphate nucleotides and rinsed in Buffer I (1M TRIS base, 20 mM $MgCl_2$, pH 7.5). The reaction was blocked by incubation for 20 minutes at 42°C in Buffer I containing 2% bovine serum albumin (BSA), followed by 10 minute Buffer I at room temperature. The preparations were then labeled with fluoresceine isotiocyanate (FITC)-conjugated anti-digoxigenin antibody (Boeringher, Mannheim, Germany), diluted 1:500 in Buffer I, 2% BSA; the incubation was performed at 37°C for 30 minutes. Nuclei were counterstained with 1 μg/ml propidium iodide (PI) and the specimens were observed by confocal microscopy. For confocal microscopic analysis, the samples were observed with a Leica TCS 4D equipped with an Argon ion laser, attached to Leitz DMRB fitted with a 100X/1.3 NA oil immersion objective. For the image acquisition, FITC and PI were excited with the blue (488 nm) and green (514 nm)line of the argon ion laser, respectively. Thereafter, serial optical sections of the FITC signal, performed on z-axis and merged with the corresponding PI images, were elaborated and reconstructed as a three dimensional image (De La Torre *et al*., 1993; Falcieri *et al*., 1994a,b; 1996).

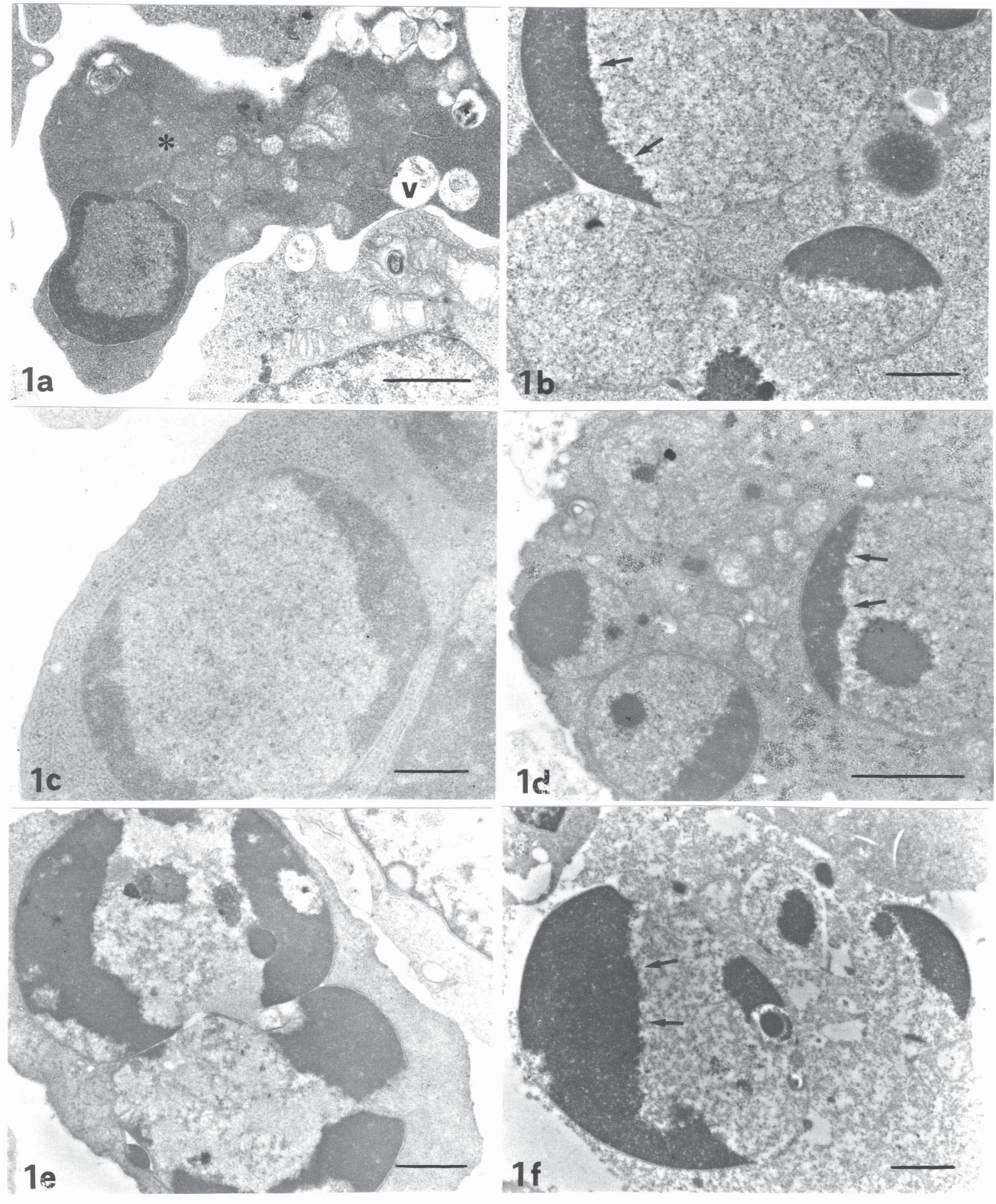
Figure 1 (facing page). Transmission electron microscopy of Molt-4 cells after 24 hour incubation with staurosporine (**a, b**), camptothecin (**c, d**) and tiazofurin (**e, f**). All apoptotic features, such as cytosol condensation (*), vacuoles (v), chromatin margination (—>), and formation of gradually condensing micronuclei appear. An increasing disruption of cytoplasm and membrane can be observed after tiazofurin treatment (**f**) (bar = 1 μm).

Results

Staurosporine treated Molt-4 cells showed the apoptotic features previously reported (Falcieri *et al*., 1993). Changes were seen in the cytoplasm and the nucleus; the latter changes mostly involved chromatin arrangement. At 24 hours, 30 to 40% of the cell population showed a characteristic condensation of the cytoplasm with a simultaneous appearance of large vacuoles (Fig. 1a); this was followed by disintegration of the nucleus into numerous micronuclei. Further chromatin condensation finally generated cap-shaped dense structures sharply separated from the diffuse chromatin areas (Fig. 1b).

Camptothecin addition induced comparable ultrastructural changes after 24 hours (Fig. 1c). In a later phase of apoptosis, cells with numerous, partially condensed, micronuclei are commonly observed (Fig. 1d). When apoptosis was induced by tiazofurin, a similar nuclear rearrangement was seen (Fig. 1e), but also a progressive swelling of the cytoplasm with signs of membrane disruption (Fig. 1f). Apoptosis induced by tiazofurin appeared to be followed by necrosis. This response to tiazofurin makes it more difficult to analyse changes in cell organelles. On the other hand, after staurosporine and camptothecin treatment, cell organelles appeared relatively well-preserved, as also described in other apoptotic models (Falcieri *et al*., 1993, 1994a,b). Some focal swelling of the cells can be observed, usually correlated to cytosol condensation, but membrane and cytoplasmic components can be recognized for long periods after apoptosis sets in.

Conventional agarose DNA electrophoresis of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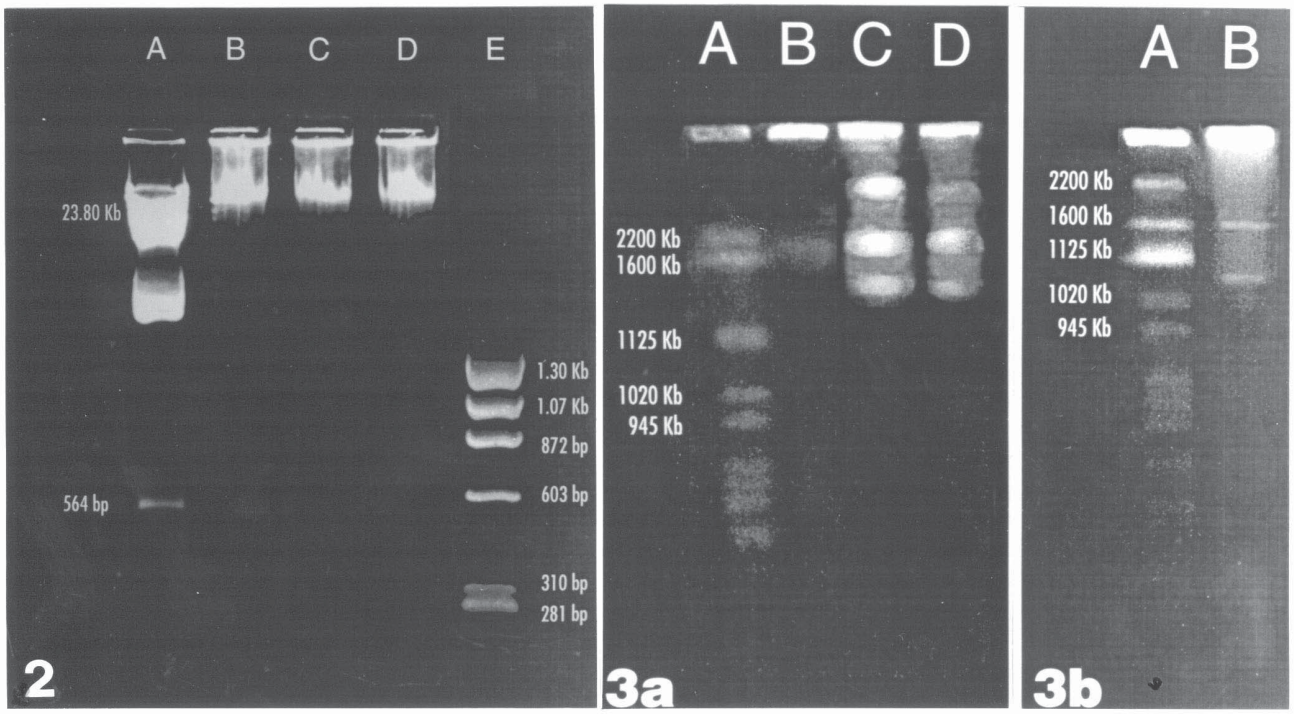

Figure 2. Agarose gel electrophoresis of DNA extracted from Molt-4 cells after 24 hours of treatment. Lane A: Marker λDNA/HInd III; lane B: DNA from Molt-4 control cells; lane C: Molt-4 incubated with 0.15 μM camptothecin; lane D: Molt-4 incubated with 10 μM tiazofurin; lane E: Marker ∅X174 DNA/Hae III.

Figure 3: Pulsed-field gel electrophoresis in 1% low melt agarose of DNA from Molt-4 cells after 24 hours of treatment. (**a**) lane A: yeast chromosomal DNA (PFGE Marker II; Boehringer, Mannheim, Germany) as molecular weight standard; lane B: Molt-4 control cells; lane C: Molt-4 incubated with 15 μM staurosporine; lane D: Molt-4 incubated with 0.15 μM camptothecin; **(b**) lane A: yeast chromosomal DNA as molecular weight standard; lane B: Molt-4 incubated with 10 μM tiazofurin.

Figure 4: (facing page) *In situ* NT labelling of Molt-4 cells after 24 hour incubation with staurosporine (**a, b**) camptothecin (**c, d**) and tiazofurin (**e, f**). The bright fluorescent spots label the single strand DNA breaking points. Diffuse fluorescence appearing in staurosporine treatment (**a**), gradually clusters towards nuclear poles (**b**). Camptothecin treatment shows large fluorescent patches (**c**) or entirely labeled micronuclei (**d**). An intermediate pattern appears after tiazofurin incubation (**e, f**) (bar = 10 μM).

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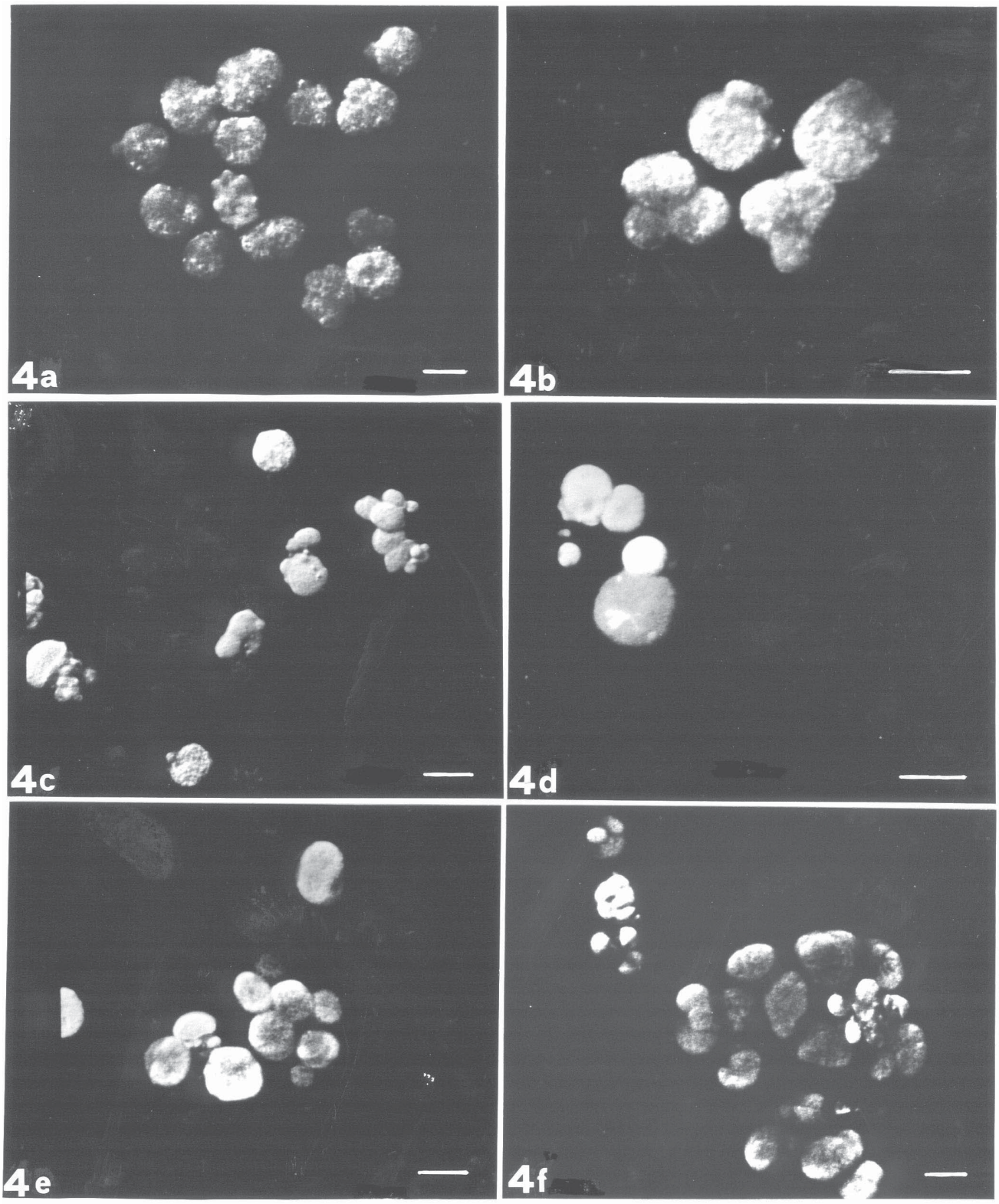
camptothecin and tiazofurin treated cells did not show the typical DNA ladder (Fig. 2). An oligonucleosomic DNA fragmentation is thus absent, as previously reported for staurosporine treatment (Falcieri *et al*., 1993).

The possible presence of larger DNA fragments was investigated by means of pulse-field gel electrophoresis (PFGE). Different runs of staurosporine, camptothecin and tiazofurin treated cell DNA, respectively, were performed, because of the large number of cells required by the technique. Bands of high molecular weight DNA were identified after all three treatments (Figs. 3a,b). In cells treated for 24 h with staurosporine and camptothecin, three high molecular weight fragments were present: > 2200 kb, 1900 kb and 1400 kb (Fig. 3a: lanes C, D). Also in the control DNA (lane B), a small amount of 1900 kb fragments can be observed. This phenomenon is seen occasionally and can be ascribed to spontaneous apoptosis, which can also be observed by electron microscopy and NT of control cells. The tiazofurin treated cells showed two fragments, one of 1600 kb and one of about 1070 kb (Fig. 3b: lane B).

When single strand DNA cleavage was analyzed, particular features were shown by the *in situ* NT technique. In this molecular approach, areas of green fluor-escence identify the spots corresponding to single strand cleaved DNA, while intact DNA, stained by propidium iodide, shows a compact red fluorescence. In black and white pictures,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the red fluorescence is generally not seen and the bright areas are considered NT positive.

In 60-70% of the cells a finely spotted nuclear fluorescence appeared after staurosporine treatment (Fig. 4a), which gradually clustered towards a nuclear pole (Fig. 4b), as previously reported in other apoptotic systems (Falcieri *et al.*, 1994a). Camptothecin treatment of cells produced a different labelling pattern, showing large fluorescent patches or entirely labeled nuclei and micronuclei (Fig.4 c,d) (Falcieri *et al*., 1994a). 25-30% cells appeared positive in this case.

Among tiazofurin treated Molt-4 cells, which showed 10-20% labelled nuclei, both staining patterns could be observed and finely spotted, as well densely fluorescent nuclei, appeared (Fig. 4e,f).

Discussion

In this study, we induced apoptosis in Molt-4 cells by means of three agents with different mechanisms of action. Staurosporine, an alkaloid isolated from Streptomyces cultures, is a potent protein kinase inhibitor with a broad spectrum of activity and generally arrests cell cycle progression preventing cells from entering the S-phase (Bruno *et al*, 1992; Bertrand *et al*., 1994). In a previous paper, we described its effect on Molt-4 cells, where, even though the conventional apoptotic features were present, conventional agarose gel electrophoresis could not demonstrate DNA fragmentation (Falcieri *et al*., 1993). The lack of activation of endonucleases can be postulated in this apoptotic model, despite the existence of a metabolic pathway responsible for chromatin rearrangement and other apoptotic patterns.

In the present study, we attempted to clarify this behavior by using other inducers of apoptosis and we analyzed their effect by several different techniques. Camptothecin, a chemically identified plant alkaloid, inhibits topoisomerase I, thus affecting the spatial arrangement of DNA (Kaufmann *et al*., 1989; Bertrand *et al*., 1994) and, consequently, DNA and RNA synthesis. The action of camptothecin is markedly different from that of staurosporine but neither in this condition, a DNA ladder can be found, despite the presence of an apoptotic ultrastructure. Therefore, also in the case of camptothecin, the typical endonuclease is not activated, but nuclear changes occur. Tiazofurin is an antitumor agent capable of inducing erythroid differentiation, and down-regulating phospholipase $C\beta_1$ (Manzoli *et al*., 1995). It selectively blocks IMPDH, an enzyme which is activated in cancer cells, and is crucial in the synthesis of guanosine triphosphate. This nucleotide is also an intermediate compound active in signal transduction, RNA synthesis and other biochemical pathways (Tricot *et al*., 1990; Weber *et al*., 1991; Manzoli *et al*., 1995). When Molt-4 cells are treated with tiazofurin, they show an apoptotic morphology very similar that induced by camptothecin and staurosporine. Conventional agarorse gel electrophoresis shows the absence of a DNA ladder.

A different type of single strand DNA cleavage is evident in the three different experimental conditions, both in terms of the percentage of cells showing a positive reaction, and in terms of the labelling pattern. Staurosporine treatment produces a finely spotted labelling of the nuclei in 60-70% cells indicating a diffuse distribution of DNA single strand breaks, which gradually cluster towards the nuclear poles and form cup-shaped positively labelled areas (Falcieri *et al*., 1994a). On the other hand, single strand breaks induced by camptothecin are present in only 25-30% of the cells, and appear clustered in large fluorescent patches, indicating both a larger number of breaks and their possible successive spatial rearrangement, presumably due to the mechanism of action of camptothecin, which affects DNA architecture (Kaufmann, 1989). Tiazofurin incubation produces a markedly lower cell labelling. Punctate labelling, as well as intensively positive patches appear, suggesting a partial spatial rearrangement of the single strand breaks, consequent to single strand cleavage.

In all apoptotic models, conventional DNA electrophoresis failed to reveal oligonucleosomic DNA fragmentation, which suggests the absence of endonuclease activity. Nevertheless, genomic DNA cleavage is detectable and reproducibly found by PFGE. Three bands, corresponding to >2200 kb, 1900 kb and 1400 kb appear after staurosporine and camptothecin treatment, while two bands at 1600 kb and 1070 kb are present after tiazofurin incubation. This difference in DNA fragment size is probably due to the different mechanism of action of the drugs used.

Staurosporine and camptothecin could, in the absence of an endonuclease, activate another enzymatic pathway in Molt-4 cells, leading to the appearance of 2200 kb, 1900 kb and 1400 kb DNA fragments. On the other hand, tiazofurin decreases the concentration of guanosine triphosphate and reduces the size of the intracellular nucleotide pool. The mechanism of action of tiazofurin is not known in detail; this substance has mainly been studied in a clinical context because of its antineoplastic activity (Tricot *et al*., 1990), but the effect on the intracellular nucleotide pool could be responsible for the different pattern of genomic cleavage, involving the formation of 1600 kb and 1070 kb DNA fragments. This unusual pattern of DNA fragmentation is intriguing and difficult to explain. Molt-4 apoptotic cells have not been widely investigated and most information on different patterns of DNA cleavage derives from basically different apoptotic models (Cohen *et al*., 1993; Oberhammer *et al*., 1993a,b, 1994; Peitsch *et al*., 1993b; Bicknell *et al*., 1994; Walker *et al*., 1995; Weaver *et al*., 1996).

In addition, we used two well-known apoptotis inducers, staurosporine and camptothecin which induce typical apoptotic patterns (also in terms of oligonucleosomic fragmentation) in other cell lines (Kaufmann, 1989; Bertrand *et al*., 1994).

Why DNA nucleosomic cleavage does not occur in Molt-4 cells is not clear. We suggest that an unknown form of DNA cleavage occurs, due to a metabolic pathway that is specific for the Molt-4 cell line. The slightly different behaviour of tiazofurin treated cells could be attributed to the same, or to a very similar enzymatic system, activated when the nucleotide pool is reduced by tiazofurin treatment. Therefore, despite the different mechanisms of action of the drugs used in this study, DNA cleavage depends on enzymatic activity in the Molt-4 cells and only high weight fragments can be observed. Also single strand DNA cleavage shows slightly different features, possibly due to spatial rearrangement of DNA subsequent to the break and which gives rise to different labelling patterns. However, single strand DNA cleavage is induced by all three apoptosis inducers used in this study.

Finally, chromatin rearrangement, as observed by electron microscopy, appears comparable to what has been described in other well known apoptotic models (Vitale *et al*., 1993; Zamai *et al*., 1993) and the three drugs investigated in the present paper produced comparable effects. In conclusion, apoptotic agents with very different mechanisms of action induce a similar response in Molt-4 cells, consisting of a genomic fragmentation and a closely comparable ultrastructural pattern. The programmed cell death in Molt-4 cells induced by the three agents used in this study may follow a pathway that is common for the three agents despite differences in the way they induce apoptosis.

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References

Arends MJ, Morris RG, Wyllie AH (1990) Apoptosis: the role of the endonuclease. Am J Pathol **136:** 593-608.

Barr PJ, Tomei LD (1994) Apoptosis and its role in human disease. Biotechnol **12**: 487-493.

Bertrand R, Solary E, O'Connor P, Kohn KW, Pommier Y (1994) Induction of a common pathway of apoptosis by staurosporine. Exp Cell Res **211**: 314-321.

Bicknell GR, Snowden RT, Cohen GM (1994) Formation of high molecular mass DNA fragments is a marker of apoptosis in the human leukaemic cell line, U937. J Cell Sci **107**: 2483-2489.

Bissonnette RP, Echeverri F, Mahboubi A, Green DR (1992) Apoptotic cell death induced by c-myc is inhibited by bcl-2. Nature **359**: 552-554.

Boe R, Gjertsen BT, Vintermyr OK, Houge G, Lanotte M, Doskeland SO (1991) The protein phosphate inhibitor okadaic acid induces morphological changes typical of apoptosis in mammalian cells. Exp Cell Res **195**: 237-246.

Bruno S, Ardelt B, Skierski JS, Traganos F, Darzynkiewicz Z (1992) Different effects of staurosporine, an inhibitor of protein Kinases, on the cell cycle and chromatin structure of normal and leukemic lymphocytes. Cancer Res **51**: 470-473.

Cohen GM, Sun XM, Snowden RT, Ormerod MG, Dinsdale D (1993) Identification of a transitional preapoptotic population of thymocytes. J Immun **151**: 566-574.

Collins RJ, Harmon BV, Gobè GC, Kerr JFR (1992) Internucleosomal DNA cleavage should not be the sole criterion for identifying apoptosis. Int J Radiat Biol **61**: 451-453.

De La Torre J, Sumner AT, Gosalvez J, Stuppia L (1992) The distribution of genes on human chromosomes as studied by *in situ* nick translation. Genome **35**: 890-893.

Ellis EE, Yuan J, Horvitz HR (1991) Mechanism and functions of cell death. Annu Rev Cell Biol **7**: 663-698.

Fady C, Gardner A, Briskin K, Shmid I, Lichtenstein A (1994) A typical apoptotic cell death induced in L929 targets by exposure to tumor necrosis factor. Proc Am Assoc Canc Res **35**: 312-316.

Falcieri E, Martelli AM, Bareggi R, Cataldi A, Cocco L (1993) The protein kinase inhibitor staurosporine induces morphological changes typical of apoptosis in Molt-4 cells without concomitant DNA fragmentation. Biochem Biophys Res Comm **193**: 19-25.

Falcieri E, Zamai L, Santi S, Cinti C, Gobbi P, Bosco D, Cataldi A, Betts C, Vitale M (1994a) The behaviour of nuclear domains in the course of apoptosis. Histochemistry **102**: 221-231.

Falcieri E, Gobbi P, Zamai L, Vitale M (1994b) Ultrastructural features of apoptosis. Scanning Microsc **8**: 653-666.

Falcieri E, Stuppia L, Di Baldassarre A, Mariani AR, Cinti C, Columbaro M, Zamai L, Vitale M (1996) Different approaches to the study of apoptosis. Scanning Microsc. **10**: 227-237.

Kaufmann SH (1989) Induction of endonucleolytic DNA cleavage in human acute myelogenous leukemia cells by etoposide, camptothecin, and other cytotoxic anticancer drugs: A cautionary note. Cancer Res **49**: 5870-5878.

Koury MJ (1992) Programmed cell death (apoptosis). Exp Hematol **20**: 391-394.

Manzoli L, Billi AM, Gilmour RS, Martelli AM, Matteucci A, Rubbini S, Weber G, Cocco L (1995) Phosphoinositide signaling in nuclei of Friend Cells: tiazofurin down-regulates phospholipase C β1. Cancer Res **55**: 2978-2980.

Oberhammer F, Fritsch G, Schmied M, Pavelka M, Printz D, Purchio D, Lasmann H, Schulte-Hermann (1993a) Condensation of the chromatin at the membrane of an apoptotic nucleus is not associated with activation of an endonuclease. J Cell Sci **104**: 317-326.

Oberhammer F, Wilson JW, Dive C, Morris ID, Hickman JA, Wakeling AE, Walker PR, Sikorska M (1993b) Apoptotic death in epithelial cells: Cleavage of DNA to 300 and/or 50 kb fragments prior or in absence of internucleosomal fragmentation. EMBO J **12**: 3679-3684.

Oberhammer FA, Hochegger K, Froschl G, Tiefenbacher R, Pavelka M (1994) Chromatin condensation during apoptosis is accompanied by degradation of lamin A + B, without enhanced activation of cdc2 kinase. J Cell Biol **126**: 827-837.

Peitsch C, Muller C, Tschopp J (1993a) DNA fragmentation during apoptosis is caused by frequent single strand cuts. Nucl Acid Res **21**: 4206-4209.

Peitsch MC, Polzar B, Stephan H, Crompton T, Mac Donald HR, Mannhertz HG (1993b) Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). EMBO J **12**: 371-377.

Sambrook J, Fritsch EF, Maniatis T (1989) Pulsed-field gel electrophoresis from molecular cloning. In: A Laboratory Manual, Vol. 1. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. pp. 650-659.

Schwartz LM (1995) The faces of death. Cell Death Diff **2**: 83-85.

Tricot G, Jayaram HN, Weber G, Hoffman R (1990) Tiazofurin: Biological effects and clinical uses. Internat J Cell Cloning **8**: 161-170.

Vitale M, Zamai L, Mazzotti G, Cataldi A, Falcieri E (1993) Differential kinetic of propidium iodide uptake in apoptotic and necrotic thymocytes. Histochemistry **100**: 223-229.

Walker PR, Pandey S, Sikorska M (1995) Degradation of chromatin in apoptotic cells. Cell Death Diff **2**: 97-104.

Weaver VM, Carson CE, Walker PR, Chaly N, Lach B, Raymond Y, Brown DL, Sikorska M (1996) Degradation of nuclear matrix and DNA cleavage in apoptotic thymocytes. J Cell Sci **109**: 45-56.

Weber G, Nagai M, Natsumeda Y, Eble JN, Jayaram HN, Paulik E, Zhen W, Hoffman R, Tricot G (1991) Tiazofurin down-regulates expression of c-Ki-*ras* oncogene in a leukemic patient. Cancer Commun **3**: 61-66.

Wyllie AH, Kerr JFR, Currie AR (1980) Cell death: Significance of apoptosis. Int Rev Cytol **68**: 251-306.

Zamai L, Falcieri E, Zauli G, Cataldi A, Vitale M (1993) The optimal detection of apoptosis by flow cytometry depend on cell morphology. Cytometry **14**: 891-897.

Discussion with Reviewers

A. Liepins: Tiazofurin gives a different genomic DNA cleavage by PFGE (Figs. 3a and 3b). This is not consistent with the main conclusion of the paper that the triggering agents may induce a common enzymatic pathway for apoptosis.

Authors: We agree that tiazofurin has a mechanism of action different from the two other triggers, which also differ from each other. Nevertheless, as we show in a paper recently submitted for publication, it is an apoptotic trigger that causes both a DNA ladder and structural changes characteristic for apoptosis. Tiazofurin gives a different genomic DNA cleavage by PFGE probably because of its different mechanism of action which may involve a depletion of the intracellular nucleotide pool. This effect, in combination with the activity of an endonuclease, could explain the difference in DNA fragment size.

K.M. Anderson: Apoptosis can be a morphologic accompaniment of physiologic cell death rather than a mechanism, or at least some believe, e.g., Schwartz (1995). A number of what are considered to be examples of programmed cell death do not express a typical apoptotic (nuclear) morphology. Please comment.

Authors: The editorial by Schwartz (1995) speculates about the existence of two types of programmed cell death, apoptotic and non-apoptotic cell death, the first, more precisely, concerning the morphological features of the latter. Due to the application of recent molecular techniques and by a multiple technique approach [e.g., transmission and scanning electron microscopy, immunocytochemistry, freeze-fracture, DNA gels, PFGE, field inversion gel electrophoresis (FIGE), nick translation (NT), TdT-mediated dUTP-biotin nick end labeling (TUNEL), different types of flow cytometry] to the study of cell death, apoptosis is becoming a more defined and characterized phenomenon (Falcieri *et al*., 1996). In the present paper a model is used in which programmed cell death reproducibly shows some of the widely described parameters typical of apoptosis, and particularly the ultrastructural features.

K.M. Anderson: What percentage of cells were trypan-positive after treatment with any of the drugs for up to 96 h?

Authors: Initially, 10-15% of the cells were trypan blue-positive. After 24 h incubation with staurosporine or camptothecin 15-20% were positive and, after 48h, 30%.

After 24 h incubation with tiazofurin, 30% of the cell population was positive and this increased to 40% after 48 h. Cell viability was not measured at 96 h, since the majority of the cells at that time undergoes a progressive secondary necrosis.

K.M. Anderson: What are the signs of membrane disruption? What were the effects on mitochondria, endoplasmic reticulum, lysosomes, and vacuolization? Do the cytoplasms resemble one another or are differences present?
Authors: The signs of membrane disruption in tiazofurin-treated cells are shown in Fig. 1f. This feature is the consequence of cytoplasmic swelling, which is also evident in the figure. Under these conditions more detailed investigations of the organelles, which can be hardly recognized, is not possible. However, in better preserved apoptotic cells (which is the case with staurosporine and camptothecin treatment) the organelles appear generally unaffected by the drugs, which has also been described for other apoptotic conditions. On the other hand, Molt-4 lymphocytic cells do not represent the best model for this kind of study, having a relatively high nucleo-cytoplasmic ratio.

K.M. Anderson: Do control nuclei show any fluorescence? Based on Fig. 3a, lane B, I would expect limited fluorescence.
A. Liepins: Please present a micrograph of control nuclei for comparison.
Authors: We regret that we are unable to include color prints in the present paper because of the increased cost of publishing. However, in control untreated cells intact DNA appears homogeneously red-stained with propidium iodide. (For further details see the color pictures in Falcieri *et al*., 1994a). No green fluorescence (indicating single strand DNA cleavage) is seen, because no single strand breaks occur in these control cells. Nevertheless, in a small number of cells a weak staining can be recognized, indicating some single strand DNA cleavage. Similarly, a positivity can be found in control DNA electrophoresis (Fig. 3a). This has frequently been described in control cells of other apoptotic models, and is related to a small fraction of the cells undergoing spontaneous apoptosis.

K.M. Anderson: Do the authors want to extend their discussion of the differences between PFGE and NT assay results in tiazofurin-treated versus camptothecin and staurosporin-treated cells? Although the latter two drugs yield a similar PFGE pattern, their NT results differ.
Authors: At present it is impossible to precisely correlate PFGE and NT data. PFGE data reveal double strand breaks and NT single strand breaks. Possibly, a comparison could be made using the terminal deoxynucleotidyl transferase reaction, which identifies "in situ" double strand DNA breaks (see Falcieri *et al*., 1996).

J. Hurle: The pattern of DNA fragmentation in fragments around 2000 kb is an unusual finding in previous studies of apoptosis using pulse-field electrophoresis. Can you discuss this observation in relation to the characteristic 700, 300 and 50 kb pattern of DNA fragmentation reported by e.g., Bicknell and Cohen (1995) or Cohen *et al*. (1994)?
Authors: The size of the fragments observed in our experimental model (i.e., >2200, 1900 and 1400 kb in staurosporine and camptothecin treated cells or 1600 and 1070 kb in tiazofurin treated cells) is considerably larger that the size given in the papers cited by the reviewer and others. However, the cells used in those publication as well as the inducers of apoptotis are different from Molt-4 apoptotic cells. Moreover, very little is known about the nuclear matrix in the Molt-4 cells, and changes in the nuclear matrix appear to be a crucial point for DNA degradation. Speculations about the role of lamins in DNA degradation are thus for the moment impossible. An unknown enzymatic pathway, possibly typical for Molt-4 cells could regulate this type of apoptosis.

J. Hurle: Can you include a kinetic analysis of the pattern of DNA fragmentation at different times after treatment? Does internucleosomal DNA degradation occur at more advanced stages of degeneration?
A. Liepins: In Methods, three time points are listed (6, 18 and 24 h) but the results presented are only for 24 h. Please comment.
Authors: The effects of different staurosporine concentrations and different times of incubation have been reported in a previous paper (Falcieri *et al*., 1993) and no oligonucleosomic DNA fragmentation was found. When incubation time is longer than 24 h, a progressive secondary necrosis appears which hampers the identification of the ladder. This is the case for all three drugs, but particularly for tiazofurin). Cell lysis must be considered the final step of this apoptotic phenomenon, and 24 h was chosen as the best condition for this study.

Reviewer IV: In the legends of Fig. 1 and Fig. 4, as well as in Results and Discussion, you mention "progressive changes" despite the fact that you report data from only one time point. Please explain.
Authors: Programmed cell death is generally studied in synchronized cells. When the apoptotic trigger is added, not all cells will start undergoing apoptosis at the same time, and therefore a progression from "early" to "late" changes can be commonly observed at a single time point. Another point to be consider4d is the sensitivity of the different techniques used, where NT is most sensitive (see also Falcieri *et al*., 1994c, 1996), whereas electron microscopy

"detects" apoptosis later. Finally, very early apoptotic markers must be analyzed with cution, because a small percentage of apoptotic cells is commonly present in the control population.

J. Hurle: Please clarify why the control yeast DNA shows remarkable differences in migration in Figs. 3a and 3b.
A. Liepins: Technically, all three inducing agents should be analyzed by PFGE on one gel.
Authors: Because of the very high number of cells used in PFGE, different runs were performed. Possible differences in experimental conditions (buffer, pH, voltage, temperature) can cause a different rate of migration in Figs. 3a and 3b). However, the comparison with a standard molecular weight DNA corrects for these differences, which therefore become irrelevant.

A. Liepins: Please give quantitative data for the three agents in order to provide evidence that these agents, at the concentration used, are equally effective in inducing programmed cell death.
Authors: The quantitative data, used for the three agents in the present study, can be found in published papers from us and others (Kaufmann, 1989; Weber *et al*., 1991; Bruno *et al*., 1992; Falcieri *et al*., 1993).