J Cancer Res Clin Oncol (1996) 122:603–612

ORIGINAL PAPER

Angela M. Otto · Renate Paddenberg
Sybilla Schubert · Hans Georg Mannherz

Cell-cycle arrest, micronucleus formation, and cell death in growth inhibition of MCF-7 breast cancer cells by tamoxifen and cisplatin

Received: 18 December 1995/Accepted: 13 March 1996

Abstract The induction of cell death along with cell-cycle arrest is one of the foremost mechanisms regulating cell growth. In the human breast carcinoma cell line MCF-7 we investigated two chemotherapeutic agents, the antiestrogen tamoxifen and the DNA-damaging drug cisplatin, for the relative contribution of these mechanisms to growth inhibition in culture. Growth kinetics and flow cytometry confirmed that tamoxifen at 1 μM acts mainly by arresting cells in the G0/G1 phase of the cell cycle. Compared to untreated controls, only a few more cells were detached from the monolayer and dead after a 5-day incubation. On the other hand, cisplatin at 1 μM did not induce the well-defined G2/M-arrest reported for other cell types, but resulted in a marked increase in the rate of cell death. A morphological feature observed, especially with cisplatin-treated MCF-7 cells, was the formation of numerous micronuclei (in up to 30% of the cells) and an increase in the number of binucleate cells (up to 20%). In both tamoxifen- and cisplatin- treated cultures, cell death appeared to occur by apoptosis, as indicated morphologically by cellular and nuclear shrinkage accompanied by DNA-condensation and ultimately the formation of DNA containing apoptotic bodies. However, no internucleosomal DNA degradation or endogenous endonuclease activity could be detected in the cells of the monolayer or in the mainly dead and detached cells of the culture supernatant. DNA fragmentation was only observed when isolated MCF-7 nuclei were incubated with exogenous endonucleases. However, as determined by reverse transcriptase/polymerase chain reaction amplification, MCF-7 cells do express the mRNA for DNase I, an endonuclease known to be involved in apoptosis. Thus, apoptosis is part of the growth-inhibitory process and occurs without apparent internucleosomal DNA fragmentation in MCF-7 cell cultures.

Key words Cell-cycle arrest · Cell death · Micronuclei · MCF-7 cells · Tamoxifen · Cisplatin · DNA fragmentation · Endonuclease

Abbreviations *FCS* fetal calf serum · *PBS* phosphate-buffered saline

Introduction

The aim of chemotherapy in cancer treatment is the induction of tumor regression, i.e. to arrest cell proliferation and induce cell death. With the growing appreciation of the role of programmed cell death (apoptosis) in the normal homeostasis of tissue and organ size, more attention has been given to the specific induction of cell death by tumor therapy (Hickman 1992).

Apoptotic cell death in tissues and in cultured cells can be morphologically recognized by cellular rounding and a characteristic condensation of the chromatin along the inner nuclear membrane. In later stages, the cytoplasm and the nucleus disintgrate into small apoptotic bodies. In many cases chromatin condensation is accompanied by internucleosomal DNA degradation, which can be visualized by electrophoresis of extracted DNA on agarose gels (DNA-ladder formation) (Wyllie et al. 1980). This specific DNA degradation is catalyzed

A. M. Otto (✉)[1] · S. Schubert[2]
Institut für Pharmazie, Universität Regensburg,
D-93040 Regensburg, Germany

A. M. Otto · R. Paddenberg · H. G. Mannherz[3]
Institut für Zytobiologie und Zytopathologie, Philipps-Universität,
D-35033 Marburg, Germany

Present addresses
[1] Institute for Biochemistry, University of Erlangen-Nürnberg,
Fahrstraße 17, D-91054 Erlangen, Germany
[2] Institut für Biologie, Abteilung Biophysik, Universität Stuttgart,
Pfaffenwaldring 57, D-70569 Stuttgart, Germany
[3] Institut für Anatomie und Embryologie, Ruhr-Universität,
Universtitätsstr. 150, D-44780 Bochum, Germany

by a Ca^{2+}/Mg^{2+}-dependent endonuclease (Wyllie et al. 1984). Recently, it was shown for rat thymocytes and lymphocytes that this endonuclease has properties indistinguishable from those of DNase I secreted by the pancreas (Peitsch et al. 1993). This enzyme is endogenous in many cell types (Polzar et al. 1994). However, a number of other candidate endonucleases have been proposed for particular cell systems such as DNase II (Barry and Eastman 1993) or Nuc 18 (Gaido and Cidlowski 1991) (for a review see Peitsch et al. 1994).

In most cases neither the mechanisms of drug-induced growth arrest and cell death nor the reasons for initial and acquired drug resistance in tumor therapy are fully understood (Kelland 1994). For example, cisplatin is effective in the treatment of certain epithelial tumors, especially those of testicular and ovarian origin, but carcinomas of the bladder, head and neck are also responsive (Eastman 1990; Kelland 1994). One of the proposed mechanisms of action is the platination of DNA, which is thought to lead to G2 arrest and ultimately to the induction of apoptotic cell death (Sorenson and Eastman 1988b). In spite of its simple chemical structure and the universal target, cisplatin is minimally effective against many other tumors. Its therapeutic value for the treatment of mammary cancers has been questionable, but it has received renewed interest in the last few years (Sledge 1992). Nevertheless, cisplatin has a growth-inhibitory effect on breast-cancer-derived cell lines in culture (Otto et al. 1991). Unravelling the reasons for the selectivity of cisplatin as well as for tumor resistance has, therefore, been the aim of numerous investigations.

Most estrogen-receptor-positive mammary tumors respond well to the antiestrogen tamoxifen (Taylor et al. 1984). The cellular action of this drug has been studied intensively in the estrogen-receptor-positive human breast cancer cell line MCF-7 in culture. Its growth-inhibitory effect is attributed to an arrest in G1/G0 phase of the cell cycle (Sutherland et al. 1984; Osborne et al. 1983; Taylor et al. 1983). Recently, a number of contradictory reports have been published concerning the induction of apoptosis in MCF-7 cells by antiestrogens. There have been claims that tamoxifen induces the appearance of low-molecular-mass DNA and changes in the cellular and nuclear morphology reminiscent of ongoing apoptosis (Bardon et al. 1987). Other authors, however, did not detect internucleosomal DNA degradation in MCF-7 cells accompanying the morphological changes after treatment with the antiestrogen toremifene (Wärri et al. 1993).

It was the aim of this study to compare the inhibitory effects of these two completely different chemotherapeutic agents, cisplatin and tamoxifen, on the proliferation of MCF-7 cells with particular emphasis on the analysis of cell-cycle arrest and induction of cell death. The MCF-7 cell line has multiple characteristics including hormone sensitivity as well as chromosomal and morphological heterogeneity which make it a suitable model system for studying the effectiveness of antiestrogens and other chemotherapeutic agents on carcinoma cells in vitro. To clarify whether these drugs induce apoptotic cell death, we also analyzed typical parameters such as the nuclear morphology, internucleosomal DNA fragmentation of the extracted DNA, and endogenous nuclease activity. Our results indicate that tamoxifen inhibited MCF-7 cell growth mainly by G1 arrest, while cisplatin did not cause the pronounced cell-cycle arrest reported for other cellular systems. In a large fraction of cells treated with cisplatin numerous micronuclei were detectable. What the drugs had in common was that they induce the nuclear morphology typical of apoptosis, but neither internucleosomal DNA degradation nor Ca^{2+}/Mg^{2+}-dependent endonuclease activity was observed.

Material and methods

Materials

cis-Diamminedichloroplatinum(II) (cisplatin) was a gift from Degussa. A stock solution was made at 1 mM in 0.15 M NaCl. Tamoxifen was dissolved at 1 mM in 70% ethanol and diluted in phosphate-buffered saline (PBS), pH 6.6.

Cell culture

For cell-culture experiments the methods were essentially those previously described (Otto et al. 1991). Briefly, the human breast cancer cell line MCF-7 (from G. Leclercq, Institute Jules Bordet, Bruxelles) was maintained in Richter's Improved Minimal Essential Medium without phenol red (Biochrom), supplemented with 10% fetal calf serum (FCS). Trypsinized cells were plated at a titer of 4×10^4/ml. Test compounds were added to cultures 1 day after plating and incubated for 5 days or the times indicated. We verified that cells were free of mycoplasm contamination by using the Boehringer detection kit.

Determination of cell number

The procedure was as described before (Butler 1984; Otto et al. 1991). Cell monolayers were washed and allowed to swell in a hypotonic buffer containing 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$. Cells were lysed by adding 5% benzalkonium chloride in 3% acetic acid. The resulting suspension of nuclei was diluted and counted in a Coulter counter.

Trypan blue staining

The proportion of dead cells in the monolayers and in the cell pellets of the supernatants was determined by adding a 0.4% trypan blue solution and counting stained and unstained cells in the light microscope.

Nuclear staining

The DNA-binding fluorescent dye Hoechst 33342 was added to the culture medium of MCF-7 cells at a final concentration of 0.5 μg/ml.

Cells were incubated for 20 min at 37°C and subsequently fixed with 4% formaldehyde before embedding in Mowiol (Hoechst, Frankfurt).

Flow cytometry of DNA fluorescence

MCF-7 cultures were harvested by trypsinizing and suspending the cells in PBS before fixing with 70% ethanol. After the cells had been taken up in PBS, cellular DNA was labelled with propidium iodide while the RNA was digested with RNase. DNA fluorescence was measured with a FACScan flow cytometer (Becton-Dickinson) and evaluated with a software program (CellFit).

Autoradiography of [^{3}H]thymidine-labelled cells

Cells growing in 35-mm dishes were incubated with 2 µC (16.7 pmol) [^{3}H]thymidine for 2 h. After washing with 0.9% NaCl, monolayers were overlayed twice with 10% trichloroacetic acid and then washed with absolute ethanol. Dishes received a layer of a chrom alum solution, which was allowed to dry before strips of film (Feinkorn Autoradiographie Stripping Platte AR10, Kodak) were layered on the cells and exposed in the dark for 10 days. After development of the attached film, dishes were stained with Giemsa staining solution. The unlabelled and labelled nuclei were counted in at least six random fields from duplicate dishes in the light microscope.

DNA extraction

DNA was extracted separately from adherent and detached cells. Detached cells were collected by centrifugation. Both cell populations were washed with PBS and then lysed in DNA extraction buffer (100 mM TRIS HCl pH 8.0, 200 mM NaCl, 5 mM EDTA, 0.2% sodium dodecyl sulfate (SDS), 0.2 mg/ml proteinase K) for 3 h at 56°C. The DNA was pelleted with isopropanol, washed with ethanol, dried and separated in 1% agarose gels in TRIS borate EDTA buffer.

Plasmid digestion assay

A 1-µg aliquot of DNA from the plasmid pSG5 was diluted in 20 µl 20 mM TRIS acetate buffer, pH 7.4, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, mixed with 3 µl sample and incubated at 37°C for 2 h. Reactions were stopped by the addition of 5 µl 5 × standard sample buffer and the plasmid was electrophoresed in 1% agarose gels in TRIS borate EDTA buffer.

Zymogram

The detection of nucleases by the zymogram technique described by Yasuda et al. (1989) was modified as follows. After the proteins had been separated by SDS/polyacrylamide gel etectrophoresis (PAGE) in 12% gels containing 50 µg/ml calf thymus DNA, nucleases were activated by soaking the gels in 40 mM TRIS HCl, pH 7.8, 5 mM $CaCl_2$, 5 mM $MgCl_2$, 3% Blotto (delipidated milk powder) for 20 h. DNA was stained with ethidium bromide (0.5 µg/ml) and visualized under UV light.

Isolation of nuclei

Isolation of nuclei was performed as described by Almendral et al. (1988). After lysis of the cells in 10 mM TRIS HCl, pH 7.6, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40 cell debris and nuclei were separated by density centrifugation using 0.7 M sucrose in 15 mM TRIS HCl, pH 7.6, 60 mM KCl, 15 mM NaCl, 2 mM EDTA, 0.5 mM EGTA, 0.65 mM dithioerythritol, 0.1% Triton X-100, 0.5 mM spermidine, 0.15 mM spermine. The nuclei fractions obtained were stored for 1–2 weeks in 50 mM TRIS HCl, pH 7.6, 5 mM KCl, 0.1 mM EDTA either on ice or mixed with one volume of glycerin at − 20°C.

DNA degradation in isolated nuclei

Isolated nuclei of MCF-7 cells were pelleted by centrifugation (10 min, 2000 *g*) and resuspended in 200 µl 40 mM TRIS HCl, pH 7.8, containing either 5 mM EDTA or 1 mM $CaCl_2$, 1 mM $MgCl_2$. After addition of the probes, the samples were incubated for 2–3 h at room temperature. Then the proteins were digested by the addition of 400 µl DNA extraction buffer (100 mM TRIS HCl, pH 8.0, 200 mM NaCl, 5 mM EDTA, 0.2% SDS, 0.2 mg/ml proteinase K) for 1 h at 56°C. The following steps were the same as described for DNA extraction.

Polymerase chain reaction and DNA sequencing

The presence of DNase I mRNA in MCF-7 cells was analyzed by polymerase-chain-reaction (PCR)-mediated amplification of reverse-transcribed mRNA using primers derived from the human DNase I sequence (Shak et al. 1990). The sense primers ('5-CTT CAA CAT CCA GAC ATT TG-3') match to position 240–259, the antisense primers (5'-ACC TCT GTG AAC CGG GAG AA-3') to position 580–599 of the DNase I sequence. Amplification was performed by the use of 1 unit Super Taq polymerase (P. H. Stehelin & Cie AG, Basel, Switzerland) in 10 mM TRIS HCl pH 9.0, 50 mM KCl, 0.01% (w/v) gelatin, 1.5 mM $MgCl_2$, 0.1% Triton X-100 during 45 cycles consisting of incubations of 1 min at 95°C, 1 min at 55°C and 1.5 min at 72°C. After the last cycle the incubation at 72°C was prolonged to 10 min. The PCR products were analyzed by agarose gel electrophoresis (1% agarose in TRIS borate EDTA buffer). The DNA bands obtained were extracted from the agarose gels (Jetsorb kit, Genomed GmbH, Bad Oeynhausen, Germany) and subsequently sequenced by the PCR-based CircumVent Thermal Cycle Sequencing Kit (BioLabs, USA) using the sense primers of the PCR reaction.

Results

Growth kinetics

To detect changes in the growth kinetics effected by tamoxifen and cisplatin, MCF-7 cells were counted over a period of 6 days. For both drugs a concentration of 1 µM was chosen, since it gave maximal growth inhibition (Otto et al. 1991). Figure 1 shows that the control cells grew exponentially with a doubling time of about 24 h. Cells treated with 1 µM tamoxifen showed no change in growth during the first 2–3 days, but thereafter were reduced in their rate of proliferation. In contrast, the growth of cells treated with 1 µM cisplatin was retarded from the beginning, and after 3 days the cell number remained constant.

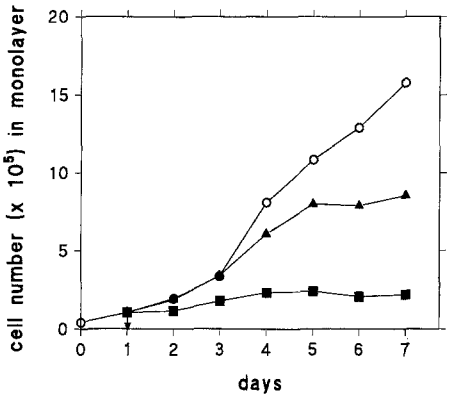

Fig. 1 Effect of tamoxifen and cisplatin on the kinetics of MCF-7 cell proliferation. (▲) Tamoxifen (1 μM) and (■) cisplatin (1 μM) were added to control cultures (○) 1 day after plating

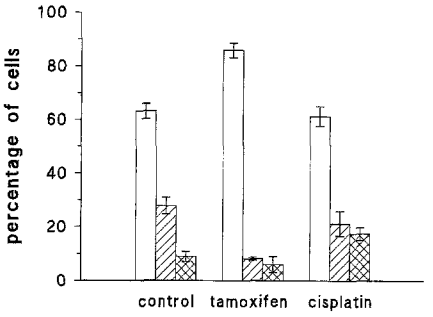

Fig. 2 Cell-cycle distribution of cells that had been treated with tamoxifen and cisplatin for 5 days as in Fig. 1. The relative DNA contents corresponding to different cell-cycle phases were determined by flow cytometry. *Open bars* G1 phase, *hatched bars* S phase, *cross-hatched bars* G2/M phase. Results are means and standard deviations of four experiments

Cell-cycle analysis

Is the reduction in the rate of cell growth due to cell-cycle arrest or cell death? To determine first the cell-cycle distribution of control and treated MCF-7 cells, the DNA content was analyzed by flow cytometry. Six days after plating, untreated cells had the typical profile of proliferating cells with 63% of the cells in G1, 28% in S, and 9% in G2/M phase (Fig. 2). Treatment with 1 μM tamoxifen for 5 days resulted in a reduction in cell number to 20% of the control. Under these conditions 86% of the cells were in G1, and only 8% and 6% in S and G2/M phase, respectively. This cell-cycle distribution is in concordance with the reported G1 arrest by tamoxifen (Taylor et al. 1983). In contrast, in cultures treated with 1 μM cisplatin, where the cell number was only 10% of the control, 62% of these cells were in G1, 21% in S, and 17% had a DNA content corresponding to the G2/M phase. Therefore, the fraction of cells with a G2/M DNA content is about twice as high as in the control culture. However, this apparent accumulation of cells in G2/M phase is not as pronounced as described for other cell systems and seems not to be sufficient to account for the observed growth arrest (Sorenson and Eastman, 1988b; Köpf-Maier et al. 1983).

To test whether the population of cells defined as being in S phase by their DNA content was actually synthesizing DNA, we determined the fraction of cells incorporating [^{3}H]thymidine during a 2-h pulse and compared it with the DNA profile obtained by flow cytometry (Table 1). In untreated cells, the labelling index was significanctly higher than the fraction of cells with S-phase DNA content. This difference is expected, since flow cytometry is the measurement of a single assay time in contrast to the radioactive labelling. After a 5-day treatment with 1 μM tamoxifen, the fraction of labelled nuclei was only about half of the fraction of S-phase cells measured by flow cytometry. Moreover,

Table 1 Determination of S-phase cells: comparison of flow cytometry and fraction of [^{3}H]thymidine-labelled nuclei. The fraction of cells in S phase after a 5-day incubation with the drugs was determined by incorporation of [^{3}H]thymidine for 2 h and by flow-cytometric assay of cellular DNA content. Data for the DNA content are taken from Fig. 2. Results are the averages of three to five experiments

Treatment	Percentage of cells with	
	[^{3}H]thymidine-labelled nuclei (%)	S-phase DNA content (flow cytometry) (%)
Control	34.3 ± 6.3	27.8 ± 3.1
Tamoxifen 1μM	5.6 ± 1.3	8.2 ± 0.6
Cisplatin 1 μM	12.9 ± 6.3	21.2 ± 4.6

compared to untreated controls, the radioactively labelled nuclei of treated cells had a smaller number of grains. This indicates a reduced rate of [^{3}H]thymidine incorporation which could be due to reduced thymidine kinase activity and/or a reduced rate of DNA synthesis. It may account for the fact that a smaller fraction of labelled nuclei was detectable in treated cultures than expected from the fraction of S-phase cells determined by flow cytometry.

A marked reduction in the labelling index compared to the fraction of cells with S-phase DNA content was likewise observed when cells were treated with 1 μM cisplatin (Table 1). Here, too, the number of grains over the labelled nuclei was markedly reduced. When these cells were allowed to continue incorporating [^{3}H]thymidine up to 24 h, the fraction of labelled nuclei increased (data not shown), which means that cells continued to progress into S phase and that the low level [^{3}H]thymidine incorporation cannot simply be ascribed to DNA repair. In a few rare cases binucleate cells were observed that had both nuclei labelled with [^{3}H]thymidine, suggesting synchronous DNA replication. The appearance of binucleate cells is in line with a defect in cell division as first reported with

cisplatin-treated cells by Rosenberg (1973) and more recently for Chinese hamster ovary cells by Rodilla et al. (1990). Nevertheless, apparently normal telophase figures were observed in the cisplatin-treated cultures. This was confirmed by immunostaining with monoclonal anti-tubulin antibody (not shown).

Nuclear morphology of drug-treated MCF-7 cells

In order to investigate the nuclear morphology and to detect changes in DNA distribution, MCF-7 cells were stained with the DNA-binding fluorochrome Hoechst 33342. Control as well as tamoxifen-treated cells had a single nucleus, oval or round in shape, with almost homogeneous staining (Fig. 3A, B). Upon cisplatin treatment the size of the nucleus increased, but was otherwise morphologically without remarkable features. However, about 20% of the cells contained two or more nuclei.

Solitary and small micronuclei were ocasionally found in about 3%–6% of untreated cells (Table 2). In tamoxifen-treated cultures, micronuclei were also observed with a similar or slightly greater frequency (Fig. 3B). Most prominent, however, was the appearance micronuclei in MCF-7 cells upon treatment with cisplatin (Fig. 3C). Not only was there a marked increase in the fraction of cells with micronuclei (Table 2), but these were also larger and more numerous per cell. Micronuclei have been observed in other cellular systems after treatment with tamoxifen (Styles et al. 1994) and with cisplatin (Rodilla et al. 1990), but have not been described for MCF-7 cells before.

In the cells of the monolayer, nuclei containing condensed DNA indicative of apoptosis were also observed (Fig. 3, Table 2). Such condensed staining was observed in cells that appeared to round-up, had a shrinking morphology and detached from the neighboring cells. These apoptotic nuclei were smaller than the normal nuclei and distinct from micronuclei, since the latter were not detectable with phase-contrast optics and were only faintly stained within the cell boundary. After a 5-day incubation, the fraction of adherent cells with apoptotic nuclei was low (in the range of 5%) even in cultures treated with tamoxifen or cisplatin.

The cultures stained with Hoechst 33342 were also used to evaluate the percentage of mitotic cells. Table 2 shows that the percentage of cells in mitosis is much lower than the total fraction of cells containing double the DNA content, depicted as G2/M phase. It can be calculated that about 25% of the cells with the G2/M DNA content were in mitosis in control cultures. In cultures treated with tamoxifen for 5 days, only about 11% of the cells in G2/M phase were in mitosis. Similarly, in cisplatin-treated cultures, the mitotic fraction of cells with G2/M DNA content was reduced to about 12%. This suggests that a few of the treated cells were still progressing through G2 into mitosis.

Kinetics of cell death

The inhibition of cell growth in cultures treated with cisplatin is not sufficiently explained either by a cell-cycle arrest or by a reduced rate in cell-cycle progression. We, therefore, also studied the contribution of cell death. In culture supernatants of growing MCF-7 cell monolayers there was always a considerable number of detached cells. These cells were stained with trypan blue in order to distinguish whether they were damaged and dead, or still viable. The kinetic analysis shows (Fig. 4) that there was a slight but steady increase in the number of detached cells even in untreated cultures, beginning about 3 days after plating, and after about 4 days 80%–95% of these detached cells were stained with trypan blue. Concomitant with the increase in detached and dead cells, there was an increase in the fraction of cells with apoptotic nuclear morphology in the monolayer (data not shown). Cells treated with tamoxifen showed no difference in this parameter, indicating that the reduced growth rate effected by

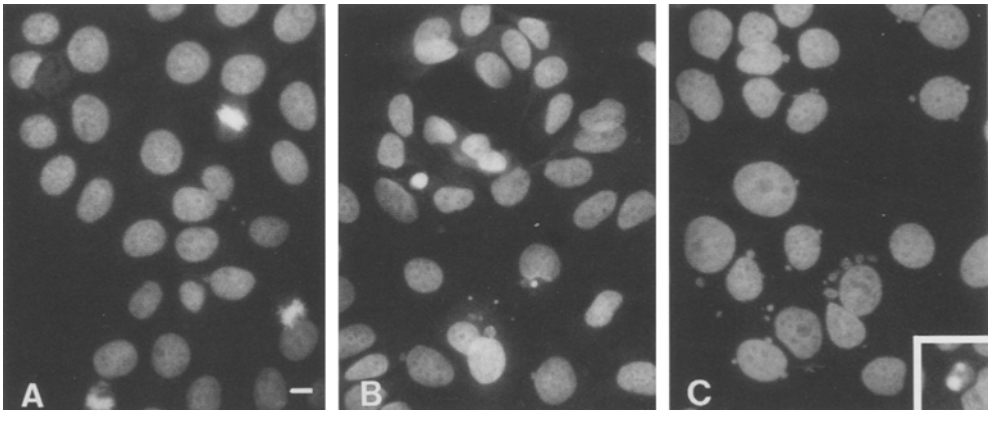

Fig. 3 A–C DNA staining with Hoechst 33342 of MCF-7 cells without treatment (**A**) and treated with 1 μM tamoxifen (**B**) or 1 μM cisplatin (**C**) for 5 days. *Insert* apoptotic cell. *Bar* 10 μm

Table 2 Comparison of the percentage of cells with G2/M-phase DNA content, with mitotic figures, micronuclei, and apoptotic morphology. Data for the G2/M phase are from Fig. 1. For the determination of cells in mitosis, with micronuclei and with apoptotic morphology, cultures were stained with Hoechst 33342 and the number of the different nuclear figures was counted. These results are the averages of one to three experiments. The number of cells with micronuclei was determined by counting 600–1100 cells each in duplicate cultures. All data were obtained after a 5-day incubation with the indicated drug

Treatment	G2/M-phase DNA content	Percentage of cells with		
		Mitotic figures (%)	Micronuclei (%)	Apoptosis (%)
Control	9.0 ± 1.9	2.1 ± 0.6	4.7 ± 1.2	0.88
Tamoxifen 1μM	6.0 ± 2.9	0.7 ± 0.3	6.4 ± 1.6	6.12
Cisplatin 1 μM	17.4 ± 2.3	1.4 ± 0.4	23.5 ± 6.5	4.50

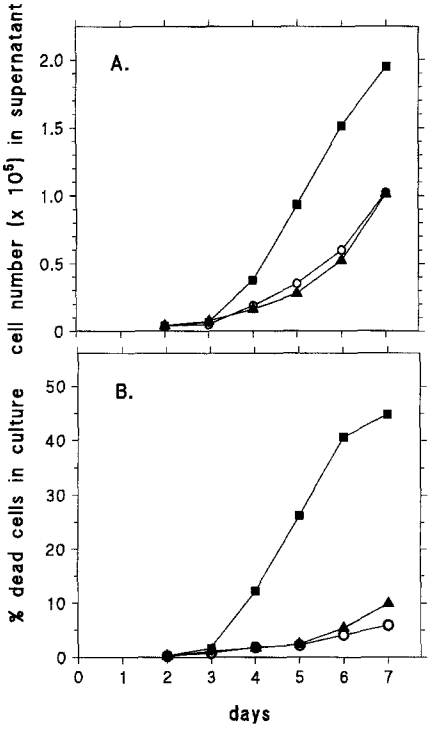

Fig. 4 **A** Kinetics of the increase in the number of detached cells in MCF-7 cultures **B** Fraction of trypan-blue-positive cells in the culture (monolayer and detached cells). (▲) Tamoxifen (1 μM) and (■) cisplatin (1 μM) were added to control cultures (○) 1 day after plating as in Fig. 1. Data from a representative experiment

tamoxifen is mainly due to cell-cycle arrest. About 10% of the total (adherent and detached) cell population in tamoxifen-treated cultures was dead on day 6, compared to 5% in control cultures. In contrast, after treatment with cisplatin there was a much greater increase in the number of cells released into the supernatant (Fig. 4A) and accordingly in the fraction of dead cells in the culture (Fig. 4B) compared to untreated cultures. Since after 3 days the cell number in the monolayer of cisplatin-treated cultures remains constant (Fig. 1), one has to conclude that the rate of cell proliferation equals the rate of cell death.

Biochemical parameters of apoptosis

Since we observed morphological evidence for apoptosis in MCF-7 cells, we looked for internucleosomal DNA fragmentation and endonuclease activity as biochemical parameters accompanying apoptosis. Gel electrophoresis of DNA extracted from adherent and detached cells of control and drug-treated cultures showed no evidence for an internucleosomal degradation pattern (Fig. 5). Furthermore, homogenates prepared from control and drug-treated cells revealed no nuclease activity in either the plasmid digestion assay (non-denaturing conditions) or zymogram analysis (denaturing conditions) (data not shown).

In isolated nuclei of the murine leukemia cell line L1210, an endogenous endonuclease can be activated by adding Ca^{2+} and Mg^{2+}, and this results in internucleosomal DNA cleavage, observed after gel electrophoresis as the typical ladder pattern (Segal-Bendirdjian and Jacquemin-Sablon 1995). To test whether the nuclei of MCF-7 cells also exhibit this endogenous nuclease activity, nuclei of control and drug-treated cultures were isolated and incubated in buffers containing either EDTA or Ca^{2+}/Mg^{2+}. The extracted DNA showed only bands of high-molecular-mass DNA; a DNA ladder indicative of the activation of endogenous nucleases was not detectable (Fig. 6, lanes 1 and 2).

To test whether the chromatin of MCF-7 cells is in principle degradable into internucleosomal fragments, nuclei isolated from untreated and drug-treated cells were incubated with exogenous Ca^{2+}/Mg^{2+}-dependent nucleases (45 to 54-kDa nucleases of mycoplasmal origin). As Fig. 6 (lanes 4) shows, the chromatin of MCF-7 cells is indeed susceptible to internucleosomal DNA cleavage and this susceptibility is not impaired in the nuclei of cells treated with the DNA-binding drug cisplatin.

Since no internucleosomal DNA fragmentation and no Ca^{2+}/Mg^{2+}-dependent nuclease were detectable in MCF-7 cells, the possibility that these cells lacked the genetic expression for such a nuclease was considered. One of the apoptotic Ca^{2+}/Mg^{2+}-dependent nucleases presumed to be involved in internucleosomal DNA degradation of various cell types is DNase I (Peitsch et al. 1993). To test whether MCF-7 cells express the mRNA for this enzyme, reverse-transcribed RNA was analyzed by PCR-mediated amplification using primers derived from the known sequence of the human DNase I (Shak et al. 1990). Analysis of the PCR products by agarose gel electrophoresis revealed two amplification products: one of about 360 bp as was expected on the basis of the matching sites of the

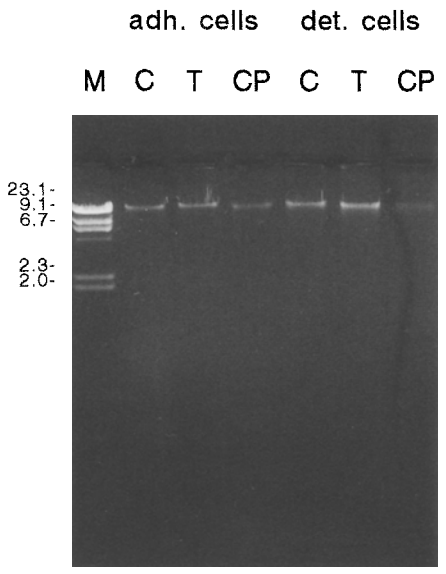

Fig. 5 Gel electrophoresis of MCF-7 cell DNA isolated separately from adherent (*adh.*) and detached (*det.*) cells of cultures incubated either without addition of drugs (*C*) or in presence of 1 μM tamoxifen (*T*) or 1 μM cisplatin (*CP*) for 5 days. *M* base-pair markers

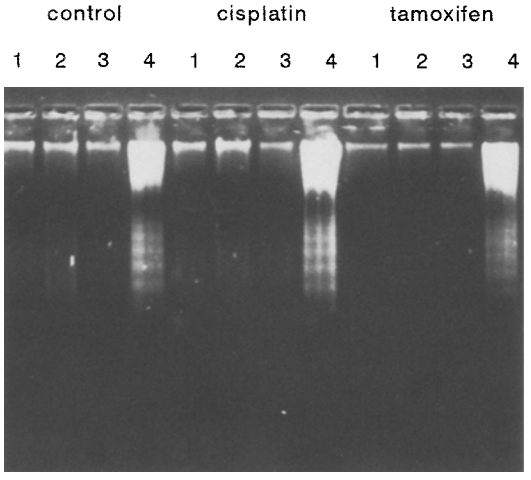

Fig. 6 DNA degradation in nuclei isolated from control, cisplatin- and tamoxifen-treated cells. Isolated nuclei were incubated in buffer containing either EDTA (*1, 3*) or Ca^{2+}/Mg^{2+} (*2, 4*), without (*1, 2*) or in the presence of (*3, 4*) mycoplasmal endonucleases before DNA extraction

primers (position 240–259 and position 580–599) and a somewhat larger product. Sequence analysis of 89 nucleotides of the 360-bp PCR product showed 100% identity to the DNase-I-specific cDNA. (The larger amplification product was not related to the DNase I cDNA, judging by the sequence analysis of 121 base pairs.) MCF-7 cells, therefore, transcribe a DNase-I-like gene, but apparently the resulting product is not active.

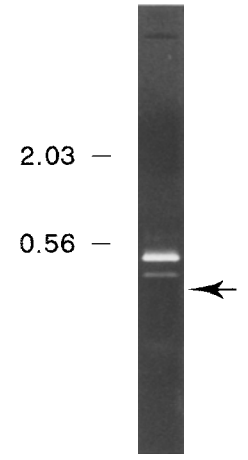

Fig. 7 Polymerase-chain-reaction (PCR)-mediated amplification of reverse-transcribed (RT) mRNA using specific primers derived from the human DNase I sequence. RT-PCR amplification products of total RNA from MCF-7 cells resulted in two products. Sequence analysis indicated that the band marked by the arrow contains a stretch of 89 base pairs identical to the DNase I cDNA. Molecular mass markers are in kbp (10^3 base pairs)

Discussion

The breast carcinoma cell line MCF-7 is a model system distinct from leukemic and other cell systems commonly used in studying apoptosis and the growth-inhibitory effects of chemotherapeutic drugs in vitro. Characteristics of this epithelial-like cell line are the high level of estrogen receptors, its sensitivity to steroid hormones (e.g. for the induction of estrogenic responses) and the morphological as well as chromosomal heterogeneity, which may thus mimic to some extent the tumor situation in vivo. This heterogeneity may also explain the selection of various sublines in different laboratories. It should be mentioned, though, that our MCF-7 cells have had a relative constant estrogen receptor level and reproducible growth behavior for the last 5 years.

It is well established that tamoxifen inhibits the growth of MCF-7 cells in culture and arrests cells in the G1 phase of the cell cycle (Taylor et al. 1984; Sutherland et al. 1984; Osborne et al. 1983). In line with these reports, we likewise observed that about 86% of the cells had arrested in the G1 phase after 5 days of tamoxifen treatment. The growth kinetics show a delay of 2–3 days before the proliferation rate was reduced (Otto et al. 1991). This is congruent with an earlier report of experiments using synchronized MCF-7 cells, which concluded that tamoxifen is effective only at a precise period in mid G1 (Taylor et al. 1983) and thus requires passage through the cell cycle. Why this early delay in the kinetics was not observed in the previous studies by others (Sutherland et al. 1983; Taylor et al. 1984; Osborne et al. 1983) can not be discerned from the available data. We observed this delay also when cells were grown in medium containing charcoal-treated serum as in the studies cited.

Our results verified earlier, indirect conclusions that growth inhibition by tamoxifen is due to cell-cycle arrest rather than to an increase in the rate of cell death

(Sutherland et al. 1983). However, after longer incubation times (5–6 days), DNA condensation indicative of apoptosis was seen in the tamoxifen-treated cells after staining with Hoechst 33342. Bardon et al. (1987) reported similar observations using electron microscopy, but cell death was detected in a higher precentage (about 20%–30%) of the attached MCF-7 cells after treatment with 100 nM–1 μM 4-hydroxytamoxifen (the active metabolite of tamoxifen). Besides cell shrinkage and chromatin condensation in the detatched cells, they also reported the appearance of low-molecular-mass DNA as a parameter of apoptosis, although these authors did not directly demonstrate DNA-ladder formation. However, Wärri et al. (1993) did not detect apoptotic internucleosomal DNA degradation in MCF-7 cells incubated with another antiestrogen, toremifene, even though they too reported apoptotic cell death as identified by time-lapse microscopy. When MCF-7 cells are inoculated into nude mice, they retain their estrogen-dependent growth. Interestingly, under these conditions estrogen withdrawal leads to DNA-ladder formation (Kyprianou et al. 1991). This result suggests some unknown interactions or signalling mechanisms between MCF-7 cells and the neighboring tissues, which cannot be conveyed in a simple cell-culture system. There is evidence that tamoxifen-treated MCF-7 cells in culture may not be able to accomplish all the processes typical of apoptosis, since certain cytoplasmic and nuclear changes differed from those of a typical apoptosis (Zakeri et al. 1995). Owing to the absence of "professional" phagocytotic cells to remove apoptotic bodies, detached MCF-7 ultimately undergo necrosis.

The mechanism of action for the growth-inhibitory effect of cisplatin has been studied in several cell types, including Hela cells (Roberts and Fraval 1978), the leukemic cell line L1210 (Sorenson and Eastman 1988b; Sorenson et al. 1990), Ehrlich ascites cells (Köpf-Maier et al. 1983), Chinese hamster ovary cells (Sorenson and Eastman 1988a), and JB1 rat hepatoma cells (Evans and Dive 1993). In these cells cisplatin was found to arrest the cell cycle in the G2 phase and to induce apoptosis, as indicated by internucleosomal DNA degradation. In contrast, our results show that in MCF-7 cells, growth inhibition by cisplatin was not accompanied by a pronounced G2 arrest. While there was an increase in the fraction of cells in G2, the majority of cells were in the G1 and S phases. The cells apparently continue to progress slowly through the cell cycle as indicated by a low [^{3}H]thymidine-labelling index and the presence of mitotic cells. There was an increase in the fraction of binucleate cells, which suggests that these cells were able to complete nuclear division, but were blocked in cytokinesis. These binucleate cells can contribute to the fraction of cells with G2/M-phase DNA content in flow cytometry. The growth kinetics of MCF-7 cells incubated with cisplatin show that after 2 days the culture sustained a constant number of adherent cells, while there was an exponential increase in the number of detached cells in the supernatant, 95% of which were dead. This means that growth inhibition was manifested by a balance between cell proliferation and cell death.

Cisplatin and, to a lesser extent, tamoxifen induced the formation of small DNA-containing vesicles. Even in untreated, exponentially growing MCF-7 cultures, a few rare cells may have a small micronucleus associated to the nucleus, suggesting that these cells may be prone to micronucleus formation. A possible explanation could be their high aneuploid DNA content. The formation of such micronuclei has been used by others as a toxicological assay for genotoxicity and mutagenesis of drugs, even though the formation and fate of the micronuclei remains obscure (Stopper et al. 1994). Tamoxifen has been reported to have genotoxic effects as shown by the formation of micronuclei in human lymphoblastoid cell lines with a frequency of about 2%–5% within 24 h (Styles et al. 1994). In Chinese hamster ovary cells cisplatin induced both micronuclei and binucleate cells (Rodilla et al. 1990; Ren et al. 1993; Rodilla et al. 1993). In double-labelling experiments with Hoechst 33342 and a monoclonal anti-tubulin antibody (data not shown), we found centrally organized microtubules with a bipolar arrangement and two distinctly separated nuclei in telophases of MCF-7 cells treated with tamoxifen or cisplatin. It can, therefore, be postulated that in MCF-7 cells the micronuclei arise from fragmentation of the nuclei by a disorganized rearrangement of the nuclear envelope after mitosis rather than by acentric chromosome segregation. However, the appearance of cells with two or more nuclei after cisplatin treatment also suggests a defect in the final phase of anaphase. A defect in the separation of nuclei has been observed in *Physarum polycephalum* upon treatment with cisplatin, which leads to polyploid nuclei (Wright et al. 1984). It has been reported that cisplatin, albeit at high concentrations, impairs microtubule assembly in vitro (Peyrot et al. 1986), and microtubule-disrupting drugs can induce the formation of micronuclei and polyploid cells (Ren et al. 1993; Majone et al. 1992; Pacchierotti et al. 1991).

The question remains: what are the underlying biochemical mechanisms involved in the death of MCF-7 cells? In cell-culture conditions, our study agrees with that of Wärri et al. (1993) showing that MCF-7 cells can display the morphological characteristics of apoptosis without internucelosomal DNA degradation following antiestrogen treatment. Moreover, upon serum removal, MCF-7 did not degrade chromatin to the larger DNA fragments of 300 kbp or to 50 kbp (Oberhammer et al. 1993). In contrast to these results, internucleosomal DNA degradation has been reported by other authors (Geier et al. 1995) for MCF-7 cells in culture treated with cycloheximide in serum-free medium. The reason for the discrepancy is not immediately apparent, but it could be explained by the selection of

a clone of MCF-7 cells different from the one used in this study. Another explanation, however, may be the recent observation that DNA fragmentation can be detected under various conditions of stress (i.e cycloheximide treatment) imposed on MCF-7 cells infected with mycoplasms (Paddenberg et al. 1996). Also, the fact that exogenous apoptotic endonuclease from another cell line can induce internucleosomal DNA degradation in MCF-7 nuclei suggests that an essential co-factor of apoptosis, which regulates DNA degradation, may be missing or inactive under normal cell-culture conditions. On the other hand, even though MCF-7 cells transcribe a mRNA which appears to be homologous to DNase I, they lack the active protein. Whether this is due to a mutation in the gene or a defect in translation is presently under investigation. Apparently, lack of endonuclease activity does not prevent cells from initiating the morphological alterations involved in apoptosis.

Acknowledgments A.M.O. was recipient of a "Habilitationsstipendium" and a research grant from the Deutsche Forschungsgemeinschaft (DFG) at the University of Regensburg. This work was also supported by the DFG (SFB 215) at the University of Marburg. It is a pleasure for us to thank Ms. Swantje Wulf for excellent technical assistance.

References

Almendral JM, Sommer D, MacDonald-Bravo H, Burckhardt J, Perera J, Bravo R (1988) Complexity of the early genetic response to growth factors in mouse fibroblasts. Mol Cell Biol 8:2140–2148

Bardon S, Vignon F, Montcourrier P, Rochefort H (1987) Steroid receptor-mediated cytotoxicity of an antiestrogen and an antiprogestin in breast cancer cells. Cancer Res 47:1441–1448

Barry MA, Eastman A (1993) Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. Arch Biochem Biophys 300:440–450

Butler WB (1984) Preparing nuclei from cells in monolayer cultures suitable for counting and for following synchronized cells through the cell cycle. Anal Biochem 141:70–73

Eastman A (1990) Activation of programmed cell death by anticancer agents: cisplatin as model system. Cancer Cells 2:275–280

Evans DL, Dive C (1993) Effects of cisplatin on the induction of apoptosis in proliferating hepatoma cells and nonproliferating immature thymocytes. Cancer Res 53:2133–2139

Gaido ML, Cidlowski JA (1991) Identification, purification, and characterization of a calcium-dependent endonuclease (NUC 18) from apoptotic rat thymocytes. J Biol Chem 266:18580–18585

Geier A, Weiss C, Beery R, Haimsohn M, Hemi R, Malik Z, Karasik A (1995) Multiple pathways are involved in protection of MCF-7 cells against death due to protein synthesis inhibition. J Cell Physiol 163:570–576

Hickman JA (1992) Apoptosis induced by anticancer drugs. Metastasis Rev 11:121–139

Kelland LR (1994) The molecular basis of cisplatin sensitivity/resistance. Eur J Cancer 30A:725–727

Köpf-Maier P, Wagner W, Liss E (1983) Induction of cell arrest at G1/S and in G2 after treatment of Ehrlich ascites tumor cells with metallocene dichlorides and cis-platinum in vitro. J Cancer Res Clin Oncol 106:44–52

Kyprianou N, English HF, Davidson NE, Isaacs JT (1991) Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. Cancer Res 51: 162–166

Majone F, Tonetto S, Soligo C, Panozzo M (1992) Identification of kinetochores and DNA synthesis in micronuclei induced by mitomycin C and colchicine in Chinese hamster ovary cells. Teratogenesis Carcinog Mutagen 12:155–166

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

Osborne CK, Boldt DH, Clark GM, Trent JM (1983) Effects of tamoxifen on human breast cancer cell cycle kinetics: accumulation of cells in early G1 phase. Cancer Res 43:3583–3585

Otto AM, Faderl M, Schönenberger H (1991) Dissociation of estrogenic and cytotoxic properties of an estrogen receptor binding platin complex in human breast cancer cell lines. Cancer Res 51:3217–3223

Pacchierotti F, Bassani B, Leopardi P, Zijno A (1991) Origin of aneuploidy in relation to disturbances of cell-cycle progression. II. Cytogenetic analysis of various parameters in mouse bone marrow cells after colchicine or hydroquinone treatment. Mutagenesis 6:307–311

Paddenberg R, Wulf S, Weber A, Heimann P, Beck LA, Mannherz HG (1996) Internucelosomal DNA fragmentation in cultured cells under conditions reported to induce apoptosis may be caused by mycoplasmal endonucleases. Eur J Cell Biol 71:105–119

Peitsch MC, Polzar B, Stephan H, Crompton T, MacDonald HR, Mannherz HG, Tschopp J (1993) Characterization of the endogenous deoxyribonuclease involved in nuclear DNA degradation during apoptosis (programmed cell death). EMBO J 12:371–377

Peitsch MC, Mannherz HG, Tschopp J (1994) The apoptosis endonucleases: cleaning up after cell death? Trends Cell Biol 4:37–41

Peyrot V, Briand C, Momburg R, Sari JC (1986) In vitro mechanism study of microtubule assembly inhibition by *cis*-dichlorodiammine-platinum(II). Biochem Pharmacol 35:371–375

Polzar B, Zanotti S, Stephan H, Rauch F, Peitsch MC, Irmler M, Tschopp J, Mannherz HG (1994) Distribution of deoxyribonuclease I in rat tissues and its correlation to cellular turnover and apoptosis (programmed cell death). Eur J Cell Biol 64: 200–210

Ren L, Zhang H, Yang J, Zhang Z (1993) A sequential study on the use of the cytokinesis-block micronucleus method in mouse splenocytes. Mutat Res 301:223–227

Roberts JJ, Fraval HNA (1978) The interaction of antitumor platinum compounds with cellular DNA in cultured cells and animal tissues: relationship to DNA cellular repair processes. Biochimie 60:869–877

Rodilla V, Pellicer JA, Pertusa J, Mothersill C (1990) Induction of micronucleated and binucleate cells in Chinese hamster ovary (CHO) cells by *cis*-diamminedichloroplatinum (II): a morphological and morphometric study. Mutat Res 241:115–124

Rodilla V, Pellicer JA, Serrano A, Pertusa J (1993) Possible relationship between micronucleated and binucleated cells induced by cisplatin in cultured CHO cells. Mutat Res 291:35–41

Rosenberg B (1973) Platinum coordination complexes in cancer therapy. Naturwissenschaften 60:399–406

Segal-Bendirdjian E, Jacquemin-Sablon A (1995) Cisplatin resistance in a murine leukemia cell line is associated with a defective apoptotic process. Exp Cell Res 218:201–212

Shak S, Capon DJ, Hellmiss R, Marsters SA, Baker CL (1990) Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. Proc Natl Acad Sci USA 87:9188–9192

Sledge GWJ (1992) Cisplatin and platinum analogues in breast cancer. Semin Oncol 19:78–82

Sorenson CM, Eastman A (1988a) Influence of *cis*-diamminedichloroplatinum(II) on DNA synthesis and cell cycle progression in excicion repair proficient and deficient Chinese hamster ovary cells. Cancer Res 48:6703–6707

Sorenson CM, Eastman A (1988b) Mechanism of cis-diamminedichloroplatinum(II)-induced cytotoxicity: role of G2 arrest and DNA double-strand break. Cancer Res 48:4484–4488

Sorenson CM, Barry MA, Eastman A (1990) Analysis of events associated with cell cycle arrest at G2 and cell death induced by cisplatin. J Natl Cancer Inst 82:749–755

Stopper H, Eckert I, Schiffmann D, Spencer DL, Caspary WJ (1994) Is micronucleus induction by aneugens an early event leading to mutagenesis? Mutagenesis 9:411–416

Styles JA, Davies A, Lim CK, Matteis F de, White INH, Yuan Z-X, Smith LI (1994) Genetoxicity of tamoxifen, tamoxifen epoxide and toremifene in human lymphoblastoid cells containing human cytochrome P450s. Carcinogenesis 15:5–9

Sutherland RL, Hall RE, Taylor IW (1983) Cell proliferation kinetics of MCF-7 human mammary carcinoma cells in culture and effects of tamoxifen on exponentially growing and plateau-phase cells. Cancer Res 43:3998–4006

Sutherland RL, Green MD, Hall RE, Reddel RR, Taylor IW (1984) Tamoxifen induces accumulation of MCF-7 human mammary carcinoma cells in the G0/G1 phase of the cell cycle. Eur J Cancer Clin Oncol 19:615–621

Taylor CM, Blanchard B, Zava DT (1984) Estrogen receptor-mediated and cytotoxic effects of the antiestrogens tamoxifen and 4-hydroxytamoxifen. Cancer Res 44:1409–1414

Taylor IW, Hodson PJ, Green MD, Sutherland RL (1983) Effects of tamoxifen on cell cycle progression of synchronous MCF-7 human mammary carcinoma cells. Cancer Res 43:4007–4010

Wärri AM, Huovinen RL, Martikainen PM, Härkönen PL (1993) Apoptosis in toremifene-induced growth inhibition of human breast cancer cells in vivo and in vitro. J Natl Cancer Inst 85:1412–1418

Wright M, Lacorre-Arescaldino I, Macquet JP, Daffe M (1984) Induction of polyploid nuclei in the plasmodium of *Physarum polycephalum* by platinum antitumor compounds. Cancer Res 44:777–783

Wyllie AH, Kerr FR, Currie AR (1980) Cell death: the significance of apoptosis. Int Rev Cytol 68:251–305

Wyllie AH, Morris RG, Smith AL, Dunlop D (1984) Chromatin cleavage in apoptosis: Association with condensed chromatin morphology and dependence on macromolecular synthesis. J Pathol 142:67–77

Yasuda T, Mizuta K, Ikehara Y, Kishi K (1989) Genetic analysis of human deoxyribonuclease I by immunoblotting and the zymogram method following isoelectric focussing. Anal Biochem 183:84–88

Zakeri Z, Bursch W, Tenniswood M, Lockshin R (1995) Cell death: programmed, apoptosis, necrosis, or other? Cell Death Differ 2:87–96