Biochemical Pharmacology, Vol. 46, No. 3, pp. 375–382, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

ADRIAMYCIN AND DAUNOMYCIN INDUCE PROGRAMMED CELL DEATH (APOPTOSIS) IN TUMOUR CELLS

ANDRZEJ SKLADANOWSKI and JERZY KONOPA*
Department of Pharmaceutical Technology and Biochemistry, Technical University of Gdańsk, Gdańsk, Poland

(*Received* 12 *November* 1992; *accepted* 4 *May* 1993)

Abstract—HeLa S_3 cells exposed to Adriamycin® and Daunomycin® for 3 hr at EC_{90} and $10 \times EC_{90}$ concentrations and incubated in drug-free medium demonstrated the characteristics of apoptosis, morphological changes and fragmentation of DNA into oligonucleosome-sized fragments. The kinetics of DNA degradation after incubation with Adriamycin did not differ significantly at the EC_{90} and $10 \times EC_{90}$ concentrations and DNA fragmentation in cells treated with both doses of Adriamycin could be observed after 12 hr of post-incubation in drug-free medium. At the EC_{90} concentration of Daunomycin, DNA fragmentation was not observed until 24 hr after drug exposure, whereas at the $10 \times EC_{90}$ concentration apoptosis-related DNA degradation was detectable as early as 3 hr after drug treatment. For both drugs, at both studied concentrations, HeLa S_3 cells were arrested in the G_2 phase of the cell cycle as can be concluded from cell size distribution studies. Additionally, the growth inhibition of HeLa S_3 cells treated with the drugs was observed at concentrations about 10 times lower than those inhibiting DNA synthesis of these cells. At lower concentrations of Adriamycin and Daunomycin (EC_{90}), apoptosis was inhibited by post-incubation with 1 μg/mL cycloheximide whereas at higher concentrations of the drugs ($10 \times EC_{90}$) there was a potentiation of cell death in cycloheximide-treated cells. The presented results suggest that apoptosis may be the process directly responsible for cell killing by Adriamycin, Daunomycin and probably other anthracyclines, in which the cytostatic effect of these compounds leads to cytotoxicity (cell death).

Anthracyclines are one of the most widely used groups of compounds in cancer chemotherapy, with proven therapeutic effect on a wide variety of tumours. The mechanism of the antitumour action of the anthracyclines is still uncertain, although an interaction with cellular DNA is believed to be a principal mechanism of their biological activity. Several alternative mechanisms have been proposed and these include the generation of reactive oxygen species producing DNA strand scission, lipid peroxidation, and disruption of cell membrane functions and integrity [for review, see 1, 2]. Metabolism of anthracyclines may also result in production of free radicals by bioreduction leading to alkylation of cellular macromolecules [3]. Additionally, interference of anthracycline antibiotics with DNA topoisomerase II has been demonstrated and proposed to be a new molecular mechanism of the cytotoxic action of these compounds [for review, see 4]. In our laboratory, it has been shown that several anthracyclines, including Adriamycin® and Daunomycin®, are able to induce interstrand DNA crosslinks following metabolic activation of these antibiotics within the cell [5]. It has been also found that DNA crosslinking by anthracyclines may be responsible for the cytotoxic activity of these antibiotics against tumour cells.†

Although much effort has been put into elucidating the biochemical action of anthracyclines, comparatively little is known about the precise mechanism(s) by which they kill cells. Adriamycin and Daunomycin along with other DNA crosslinking agents are known to induce, as their first biological effect, arrest in the G_2 phase of the cell cycle [6]. Recently, it has been found that Adriamycin induces rapid cell death that has the morphology of apoptosis in murine intestinal crypts [7, 8].

In the present study we examined whether Adriamycin and Daunomycin induce programmed cell death or apoptosis in HeLa S_3. We studied also the possible relation between DNA-directed action of the anthracyclines and cell death induced by these compounds.

MATERIALS AND METHODS

Chemicals. Adriamycin and Daunomycin were from Farmitalia Carlo Erba (Milan, Italy). Agarose electrophoresis grade was from BDH Chemicals (Poole, U.K.); proteinase K and RNAse A were from Serva (Heidelberg, Germany); methyl-[^{3}H]-thymidine (2 Ci/mmol) was from Amersham (U.K.). pBR322 DNA *Hinf*I digests were kindly provided by Dr J. Kur (Department of Microbiology, Technical University of Gdańsk). All other reagents were of analytical grade either from the Sigma Chemical Co. (St Louis, MO, U.S.A.) or from the local purchaser.

* Corresponding author: Prof. Jerzy Konopa, Department of Pharmaceutical Technology and Biochemistry, Technical University of Gdańsk, Majakowskiego St 11, 80-952 Gdańsk, Poland. Tel. (48) 58 471516; FAX (48) 58 472694.

† A. Skladanowski and J. Konopa, submitted for publication.

Cell culture and media. HeLa S_3 cells, the media and foetal calf serum were from Gibco Europe (Paisley, U.K.). Antibiotics were from Serva. HeLa S_3 cells were grown in monolayer culture in minimal essential medium supplemented with 5% foetal calf serum and antibiotics (streptomycin, 100 μg/mL; penicillin, 100 U/mL). The cells were grown at 37° in a humidified 5% CO_2–95% air atmosphere.

Incubation with drugs. Cultures of cells in their exponential phase of growth (approximately 2×10^6 cells/10 mL) were treated with various concentrations of Adriamycin and Daunomycin for 3 hr. The drugs were added from freshly prepared stocks in 50% ethanol. Following incubation with the drugs, cell monolayers were washed twice with drug-free medium, fresh medium was added and incubation was continued for up to 72 hr. To assess the effect of protein synthesis inhibition on the induction of apoptosis, cells treated with the drugs were post-incubated in medium containing 1 μg/mL cycloheximide.

Cytotoxicity assay. The cytotoxic activity of Adriamycin and Daunomycin against HeLa S_3 cells was determined after 3 hr treatment with the drugs. Briefly, exponentially growing cells were seeded at a concentration of 1.5×10^4/mL (4 mL/tube) and incubated at 37° for 24 hr before treatment. The growth medium was then replaced with a drug-containing one and the cells were incubated for 3 hr at 37°. After incubation with the drugs, the medium was withdrawn, the cells were washed twice, 4 mL of fresh medium was added to each tube and incubation was carried out for an additional 72 hr. The concentrations of anthracyclines required for 50% and 90% growth inhibition (EC_{50} and EC_{90} concentrations) compared to control non-treated cells were determined as described earlier [9].

Inhibition of [^{3}H]thymidine incorporation into DNA. The synthesis of DNA was measured by amount of radiolabelled thymidine incorporated into trichloroacetic acid (TCA)-insoluble material derived from treated cells, compared with untreated controls. Cells (4 mL, 2×10^6 cells) were treated with the drugs for 3 hr and during the last hour of incubation radiolabelled thymidine was added (final concentration 2 μCi/mL). Following incubation, triplicate samples (0.5 mL) were mixed 1 : 1 with cold 15% TCA and left at 0–4° for 0.5 hr. The precipitated material was collected by filtration onto GF/C filters (Whatman, U.K.). The filters were washed three times with 20 mL of cold 5% TCA, followed by two washes with ethanol. The filters were then dried and their radioactivity was counted in standard toluene scintillation fluid on a Beckman LS3801 scintillation counter.

Morphological examinations. Cell morphology was evaluated by light microscopy. The cells were trypsinized, washed twice with cold saline, fixed with 5% formaldehyde for 1 hr and stained with May–Grünwald–Giemsa. Cell viability was assessed by Trypan blue dye exclusion.

Analysis of DNA fragmentation. Following treatment with drugs and the post-incubation period, cells were trypsinized, harvested by centrifugation and washed twice with ice-cold saline. DNA fragmentation in cells undergoing apoptosis was assayed according to Bertrand *et al.* [10] with minor modifications. Briefly, cells (1×10^6) were lysed in 20 μL lysing buffer containing 10 mM Tris–HCl, 10 mM EDTA, 10 mM NaCl, 0.5% SDS, pH 7.4 and 0.5 mg/mL proteinase K. After 1 hr at 50°, 5 μL of 1 mg/mL RNAse A was added to each sample and incubation at 50° was continued for 2 hr. Following incubation, the salt concentration (NaCl) was raised to 1 M, and tubes were shaken vigorously. Samples were centrifuged (30 min, 500 *g*). Supernatants were collected, 2.5 vol. of ethanol (95%) was added and DNA was precipitated overnight at −20°. The DNA pellet was resuspended in 20 μL of 10 mM Tris–HCl, 15 mM NaCl, 1 mM EDTA, pH 7.4, and separated by electrophoresis (16 hr, 1 V/cm in 1.8% agarose gel with 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8 as a running buffer). DNA was visualized by ethidium bromide staining (0.5 μg/mL, 1 hr), destained overnight in redistilled water and photographed under UV illumination. DNA of plasmid pBR322 DNA *HinfI* digests was used to estimate the size of DNA fragments.

Size distribution studies. The cell size distribution curves were generated by counting the cells on a model ZB Coulter Counter according to cell size. Briefly, 1 mL of cell suspension (0.4×10^6/mL) was further diluted with 19 mL of Isoton and counted with the lower threshold set to 5 and the upper threshold to 10; both thresholds were incremented by 5 for each count.

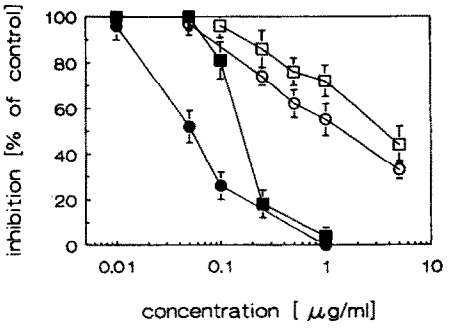

Fig. 1. The effect of Adriamycin (squares) and Daunomycin (circles) on the survival (filled symbols) and [^{3}H]thymidine incorporation (open symbols) into DNA of HeLa S_3 cells. The cells were treated with the drugs for 3 hr and cytotoxicity and [^{3}H]thymidine incorporation were determined as described in Materials and Methods.

RESULTS

The growth inhibition curves as well as inhibition of thymidine incorporation curves obtained for HeLa S_3 cells after exposure to Adriamycin and Daunomycin are shown in Fig. 1. The reduction in survival of cells incubated with Adriamycin and Daunomycin was observed at concentrations about 10 times lower than those inhibiting DNA synthesis ($EC_{50} = 0.28$ and 0.10 μM for cell growth versus $IC_{50} = 2.61$ and 1.72 μM for [^{3}H]thymidine incor-

poration, for Adriamycin and Daunomycin, respectively). Two concentrations of the drugs were used in our studies and corresponded to cytotoxic concentrations (EC_{90} values) and those inhibiting DNA synthesis as well as inducing DNA crosslinking in HeLa S_3 cells ($10 \times EC_{90}$ values) [5].

The cells treated with both anthracyclines were subjected to light microscopy following staining with Giemsa. These studies revealed that the dying cells were considerably smaller than control ones. The shrunken cells had basophilic nuclei with nuclear granules and compacted chromatin to form circumscribed masses and sometimes semilunar clumps, with intact plasma membranes (Fig. 2). All these morphological features have been defined previously as being characteristic of cells undergoing apoptosis [11]. The cells with compacted chromatin remained viable as assayed by Trypan blue dye exclusion. Only occasional necrotic cells were observed.

We next studied patterns of DNA fragmentation in HeLa S_3 cells following 3 hr exposure to Adriamycin and Daunomycin. We have found that both anthracyclines induced apoptosis-like DNA fragmentation following treatment with both studied concentrations (Figs 3 and 4). The detectable internucleosomal DNA fragmentation with characteristic "DNA ladder" was observed for Daunomycin at both 0.64 and 6.4 μM concentrations (Fig. 3, Panels B and D) and for Adriamycin at the lower concentration (0.98 μM) (Fig. 4, Panel B). At the higher concentration of Adriamycin (9.8 μM), there was marked intracellular DNA degradation which make it difficult to distinguish "DNA ladder". For Adriamycin, the kinetics of DNA degradation did not differ significantly at the two concentrations, and DNA degradation in cells incubated with Adriamycin at 0.98 and 9.8 μM occurred after 12 and 6 hr of post-incubation, respectively (Fig. 4, Panels B and D). In the case of Daunomycin, at 0.64 μM internucleosomal DNA cleavage was not evident before 12 hr of post-incubation in drug-free medium indicating that by this time the cells had not yet entered apoptosis (Fig. 3, Panel B). In contrast, at 6.4 μM of Daunomycin DNA fragmentation occurred as early as 3 hr after addition of the drug (Fig. 3, Panel D).

We analysed changes in cell size of HeLa S_3 cells treated with Adriamycin and Daunomycin using a Coulter Counter. The obtained results were similar for both drugs and we present data for Daunomycin only. At both studied concentrations of Daunomycin, the first detectable change was an increase in median cell size (Fig. 5). The initial increase was followed by a decrease in cell size by about 48 hr. During the first 12 hr, the median cell size of cells treated with Daunomycin at both concentrations approximately doubled compared to control cells.

We observed during microscopical examination and cell size studies that dying cells were smaller than control ones. We determined therefore the time course of appearance of shrunken or "apoptotic" cells in the cell population using a Coulter Counter. After treatment with the drugs and prolonged post-incubation in drug-free medium, the median size of the treated cells shifted from channel 5 (median cell size for control cells) to channel 2 (median cell size of dying cells). The results of these studies are presented in Fig. 6. At both concentrations studied of either Adriamycin or Daunomycin, there was an increase in the fraction of "apoptotic" cells with time of incubation in drug-free medium; however, the kinetics of the process differed for the two drugs. For Adriamycin, the fraction of smaller "apoptotic"

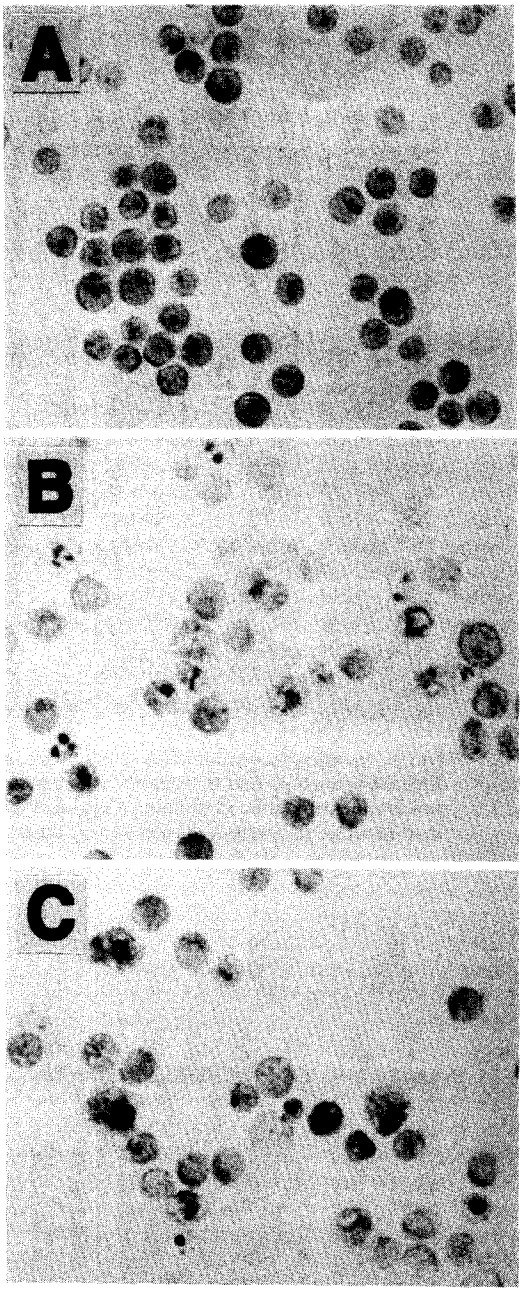

Fig. 2. Morphology of HeLa S_3 cells from untreated cultures (Panel A) and from cultures treated with either 0.98 μM Adriamycin (Panel B) or 0.64 μM Daunomycin (Panel C) for 3 hr and post-incubated in drug-free medium for 12 hr. Following incubation cells were fixed and stained with Giemsa. Objective × 40.

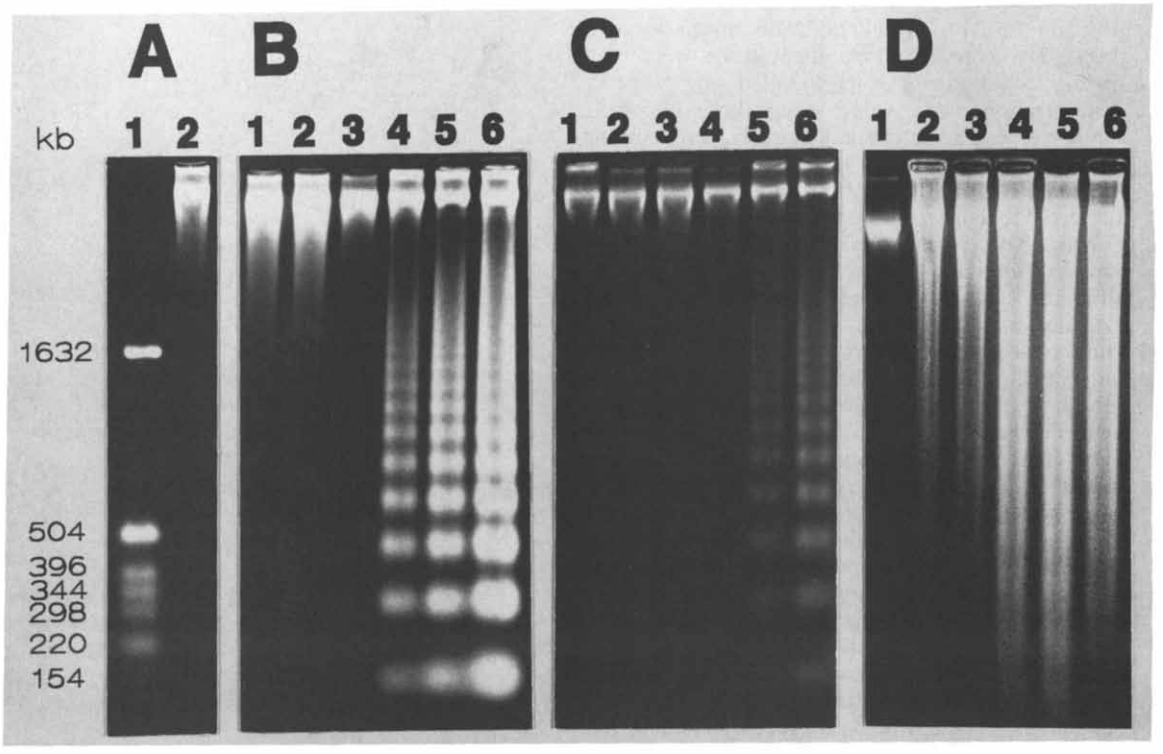

Fig. 3. Apoptosis-associated DNA fragmentation in HeLa S_3 cells treated with Adriamycin for 3 hr at 0.98 μM (Panels B and C) and 9.8 μM (Panel D) and post-incubated in drug-free medium (Panels B and D) or in medium containing 1 μg/mL cycloheximide (Panel C) for the time indicated. Panel A: lane 1, molecular weight marker (*Hinf*I digest of pBR322 DNA); lane 2, DNA from untreated cells.

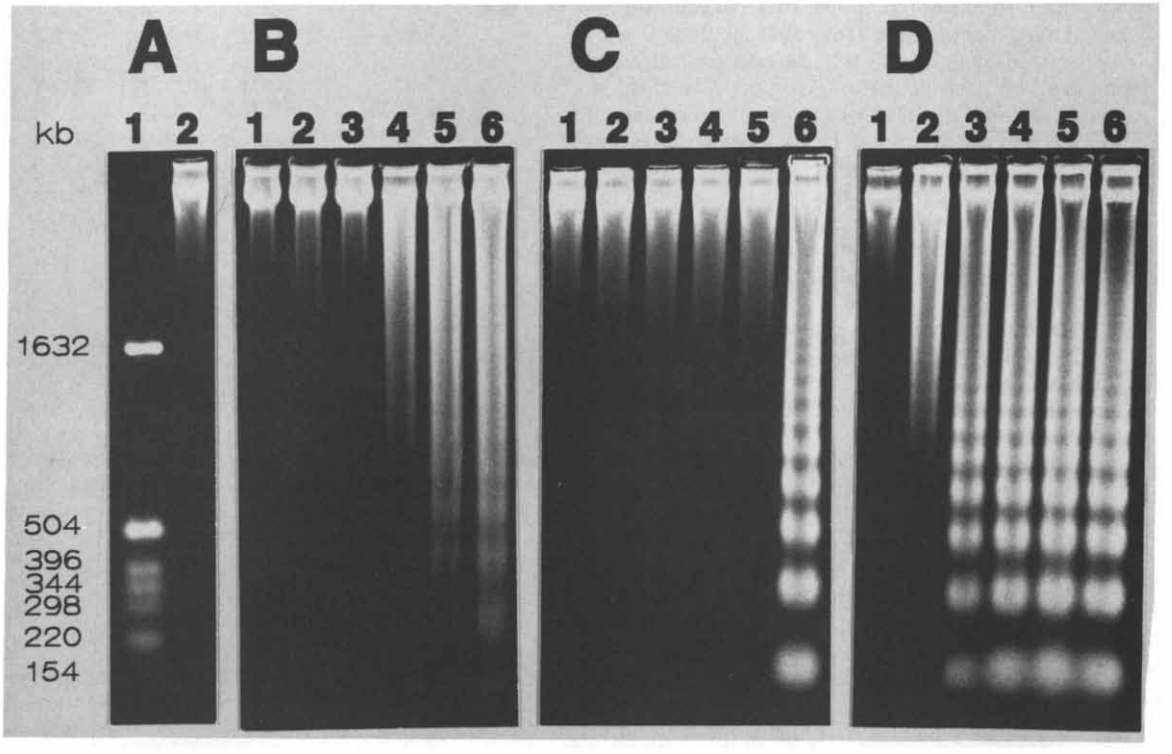

Fig. 4. Apoptosis-associated DNA fragmentation in HeLa S_3 cells treated with Daunomycin for 3 hr at 0.64 μM (Panels B and C) and 6.4 μM (Panel D) and post-incubated in drug-free medium (Panels B and D) or in medium containing 1 μg/mL cycloheximide (Panel C) for the time indicated. Panel A: lane 1, molecular weight marker (*Hinf*I digest of pBR322 DNA); lane 2, DNA from untreated cells.

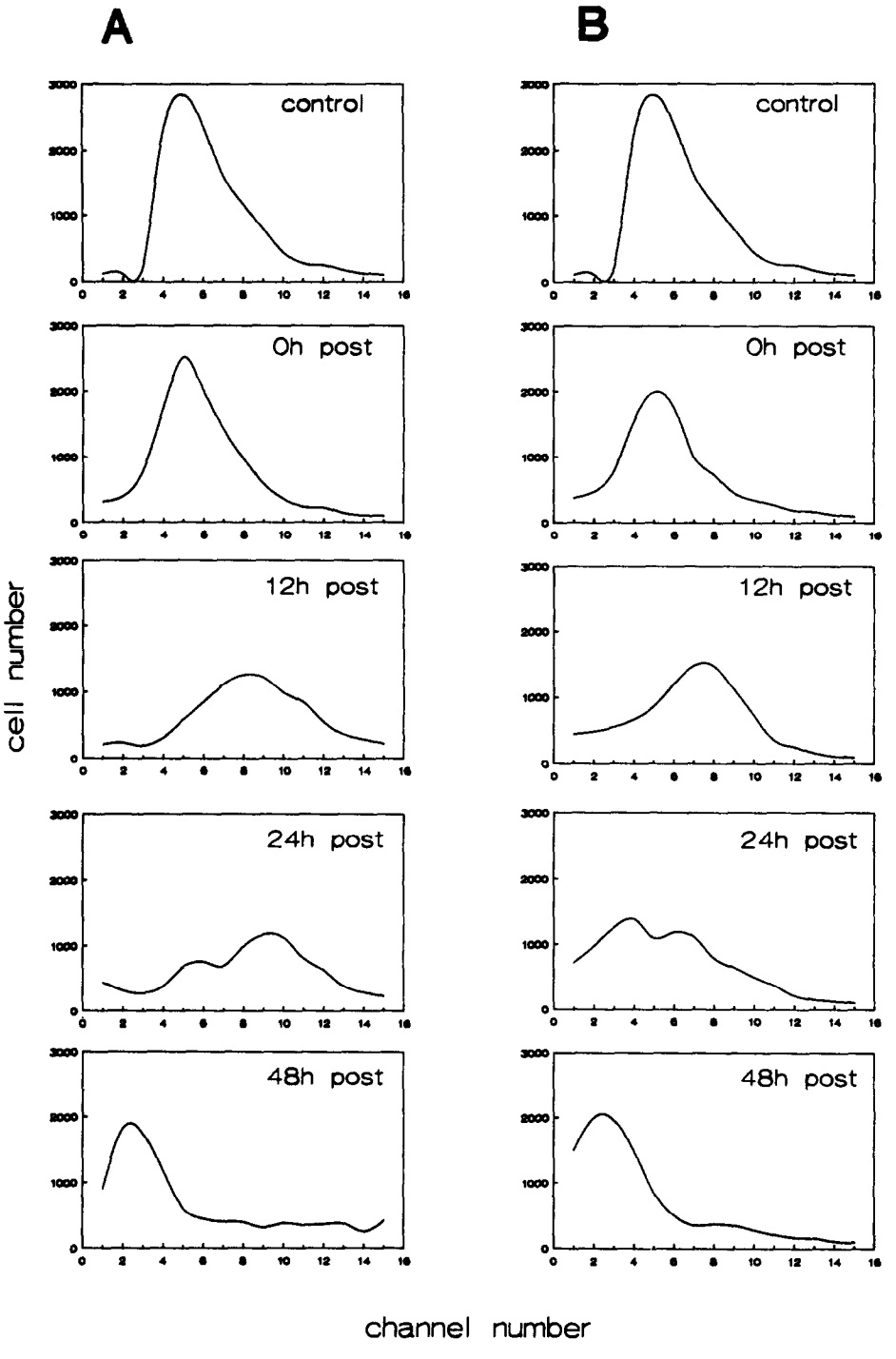

Fig. 5. Changes in the size of HeLa S_3 cells incubated with Daunomycin at 0.64 μM (Panel A) and 6.4 μM (Panel B). Coulter profiles were determined using cells treated with Daunomycin for 3 hr and post-incubated in drug-free medium for the time indicated.

cells did not increase until 24 and 12 hr had elapsed after 3 hr treatment with the drug at 0.98 and 9.8 μM, respectively (Fig. 6, Panel A). In the case of Daunomycin, there was an increase in the fraction of shrunken cells after 24 and 16 hr after 3 hr exposure of HeLa S_3 cells to the drug at EC_{90} and $10 \times EC_{90}$ concentrations, respectively (Fig. 6, Panel B). At both concentrations of the drugs, by 48 hr after drug removal the fraction of shrunken cells had reached its peak value, i.e. about 25% of the total

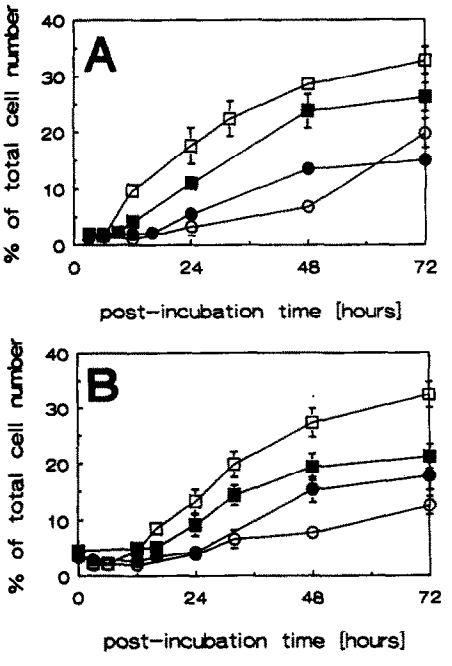

Fig. 6. Time course of the appearance of shrunken or "apoptotic" HeLa S_3 cells following 3 hr treatment with Adriamycin (Panel A) and Daunomycin (Panel B) at EC_{90} (circles) and $10 \times EC_{90}$ (squares), and post-incubation in drug-free medium (filled symbols) or in medium containing 1 μg/mL cycloheximide (open symbols). The number of apoptotic cells was determined by counting in channel 2 (peak fraction of shrunken cell population) using a Coulter Counter. Values are means from three experiments ± SD.

cell number, except the lower concentration of Adriamycin (0.98 μM) at which the fraction of "apoptotic" cells in the cell population did not exceed 15%. More than 48 hr after treatment with the drugs, we observed only decrease in total cell number.

In parallel experiments, we studied the effect of protein synthesis inhibition by cycloheximide on the morphology, DNA fragmentation and shrinkage of HeLa S_3 cells treated with both anthracyclines. We found that cycloheximide delayed apoptosis only at the lower concentration of anthracycline, i.e. EC_{90} by about 12 hr (Figs 3 and 4, Panel C). At $10 \times EC_{90}$ concentration, we observed a potentiation of cell death induced by the two drugs with unchanged kinetics (Fig. 6).

DISCUSSION

In the course of this study, we investigated whether programmed cell death is induced in HeLa S_3 cells treated with two anthracyclines, Adriamycin and Daunomycin, at concentrations corresponding to the cytotoxic activities of the drugs (EC_{90} values) and concentrations at which inhibition of DNA synthesis and DNA–DNA crosslinking could be observed* ($10 \times EC_{90}$). Treatment of HeLa S_3 cells with the two concentrations of Adriamycin and Daunomycin for 3 hr resulted in induction of DNA fragmentation. DNA cleavage into nucleosome-sized particles was observed as early as 3 hr after treatment with Daunomycin at 6.4 μM and 6 hr after treatment with Adriamycin at 9.8 μM (Figs 3 and 4). Recently, it has been reported that cyanomorpholinyldoxorubicin [12] as well as a novel *N*-(5,5-diacetoxypentyl) analogue of doxorubicin [13] are able to induce time-dependent DNA fragmentation. Both of these compounds are able to produce DNA crosslinks but, in contrast to Adriamycin and Daunomycin, without metabolic activation within the cell. The kinetics of DNA fragmentation observed for this anthracycline were very similar to those found by us for Adriamycin and Daunomycin.

Microscopic examination of cells treated with both studied anthracyclines revealed that exposure of HeLa S_3 cells to Adriamycin and Daunomycin resulted in characteristic condensation and granulation of nuclear chromatin as well as cell shrinkage, markers of apoptosis. Trypan blue staining showed that cells with fragmented nuclei were still viable, suggesting that morphological changes preceded cell death defined by dye exclusion. Cell size distribution analyses showed that the cells treated with anthracyclines initially increased in median size, followed by a decrease. The increase in median cell size suggests that anthracycline-treated HeLa S_3 cells were arrested in the G_2 phase. This observation is in agreement with the previous flow cytometry studies on the effects of Adriamycin, Daunomycin and other anthracyclines on the cell cycle of HeLa S_3 cells and other tumour cells [6 and references therein, 14]. The appearance of shrunken cells as a result of drug treatment may be related to induction of the apoptotic process. The kinetics of the appearance of shrunken or "apoptotic" cells differed depending on the concentration of the two anthracyclines applied. The difference in the kinetics of the appearance of "apoptotic cells" can be attributed to the different effects of the two doses of the drugs on traverse of the cell cycle. At a higher concentration of the drugs, the cells are probably blocked earlier in the cell cycle. Therefore, it is possible that, as was proposed for *cis*-platinum [15], at very high concentrations of the drugs cells die in all phases of the cell cycle.

We have demonstrated here that the apoptotic process was delayed by cycloheximide only at the lower concentrations of anthracyclines studied. DNA fragmentation and cell shrinkage were evident about 12 hr later than without cycloheximide treatment. At higher doses, cycloheximide potentiated cell death induced by the two anthracyclines. These results are in line with those obtained by Thakkar and Potten [8] concerning decreased toxicity of Adriamycin toward cells in which protein synthesis had been inhibited by cycloheximide. It has also been found that in addition to its protective role against cell death induced by divergent stimuli [16], cycloheximide induces apoptosis by itself [17]. The potentiation observed by us of cell death induced by Adriamycin and Daunomycin in cells treated with higher concentrations of the drugs ($10 \times EC_{90}$) and

* A. Skladanowski and J. Konopa, submitted for publication.

post-incubated with cycloheximide can be explained by the latter feature of this agent.

The higher concentrations of Adriamycin and Daunomycin applied in these studies (9.8 and 6.4 μM, respectively) are beyond the range in which these drugs stabilize a cleavable complex between topoisomerase II and DNA, when tested in a cell-free system [18]. In these studies, the concentrations of Adriamycin higher than 4.6 μM, and of Daunomycin higher than 0.95 μM, were found to inhibit topoisomerase II-related DNA cleavage. We note, therefore, that Adriamycin and Daunomycin induce G_2 block and apoptosis at concentrations at which topoisomerase II-related DNA damage is probably inhibited. This is in agreement with recent findings which have shown that the presence of stabilized DNA–topoisomerase II complexes is not essential either to the S and G_2 arrest or the induction of apoptosis by topoisomerase II inhibitors [10, 19, 20]. It has been also found that decreased toxicity of Adriamycin in the presence of cycloheximide, observed in murine intestine *in vivo*, does not appear to be through inhibition of the DNA cleavable complex by this drug [8].

The results of this study clearly indicate that direct inhibition of DNA synthesis is not a prerequisite for the cytotoxic and presumably antitumoural activity of Adriamycin and Daunomycin (Fig. 1). This is in accordance with previous reports [21, 22] as well as the mechanism of action of DNA crosslinking drugs postulated earlier [6]. Our results suggest that the studied anthracyclines exert, as their first biological effect, only cytostatic action (inhibition of cell proliferation) which is then transformed by unknown mechanism(s) into cytotoxic action (cell killing). This feature distinguishes these drugs from directly acting cytotoxic compounds such as, for example, antimetabolites.

Adriamycin and Daunomycin have been found to induce G_2 block in tumour cells *in vitro* [6 and references therein]. We hypothesized previously that antitumour compounds, which are capable of crosslinking DNA in tumour cells, when applied at optimal doses *in vivo*, arrest tumour cells in the G_2 phase [6]. We may assume that both studied anthracyclines kill tumour cells under *in vivo* conditions in the same way as they kill tumour cells cultured *in vitro*, that is by the induction of G_2 block followed by apoptosis. It follows that an antitumour effect of Adriamycin and Daunomycin could be obtained at a low dosage range which is enough for suppression of cell proliferation due to the induction of G_2 block and killing the cells by apoptosis. Such a low dosage regime would diminish unfavourable toxic effects observed after administration of both Adriamycin and Daunomycin at high doses inhibiting DNA synthesis and arresting tumour cells in the S phase of the cell cycle. If this hypothesis was true it would have an important implication for the application of anthracyclines in clinical practice. In partial agreement with this, it was found recently that Adriamycin induced apoptosis in murine intestine epithelium cells *in vivo* [7, 8]; however, inhibition of progression through the cell cycle did not appear to be necessary for the expression of apoptosis, although G_2 block was observed for these Adriamycin-treated cells [8].

In conclusion, incubation of HeLa S_3 cells with Adriamycin and Daunomycin at both cytotoxic and crosslinking concentrations induced apoptosis-associated DNA fragmentation and apoptosis-related cell shrinkage. There were differences in the kinetics of the apoptotic process between Adriamycin and Daunomycin; however, at both studied concentrations of the drugs, the end effect of their cytotoxic action was the same, namely apoptosis. The fact that both studied anthracyclines induced apoptosis at EC_{90} concentrations, i.e. at concentrations inhibiting cell growth and lower than those required for inhibition of DNA synthesis, is evidence for inhibition of DNA synthesis not being a prerequisite for the antiproliferative effect of the cell killing by Adriamycin and Daunomycin. It emphasizes also that the cytostatic effect of the anthracyclines' action (G_2 block) is converted by unknown mechanism(s) into cell killing by apoptosis. The sequence of events leading from DNA crosslinking to cellular death by apoptosis remains to be established. Further studies are planned to define the relationship between DNA crosslinking, G_2 arrest and programmed cell death induced by anthracyclines.

Acknowledgements—This work was supported by the Polish National Council for Scientific Research Grant No. 4 0109 9101 (1992). The authors are grateful to Dr B. Zurawska-Czupa and Dr M. Cichorek from the Department of Histology, Laboratory of Cytophysiology, Medical Academy of Gdańsk for help with the morphology studies.

REFERENCES

1. Powis G, Anthracycline metabolism and free radical formation. In: *Metabolism and Action of Anti-cancer Drugs* (Eds. Powis G and Prough RA), pp. 211–260. Taylor and Francis, London, 1987.
2. Tritton TR, Cell surface actions of Adriamycin. *Pharmacol Ther* **49**: 293–309, 1991.
3. Gaudiano G and Koch TH, Redox chemistry of anthracycline antitumor drugs and use of captodative radicals as tools for its elucidation and control. *Chem Res Toxicol* **4**: 2–16, 1991.
4. Potmesil M, DNA topoisomerase II as intracellular target in anthracycline treatment of cancer. In: *Anthracycline and Anthracenenedione-based Anticancer Agents* (Ed. Lown JW), pp. 447–466. Elsevier Science, Amsterdam, 1988.
5. Konopa J, Adriamycin and Daunomycin induce interstrand DNA crosslinks in HeLa S_3 cells. *Biochem Biophys Res Commun* **110**: 819–826, 1983.
6. Konopa J, G_2 block induced by DNA crosslinking agents and its possible consequences. *Biochem Pharmacol* **37**: 2303–2309, 1988.
7. Anilkumar TV, Sarraf CE, Hunt T and Alison MR, The nature of cytotoxic drug-induced cell death in murine intestinal crypts. *Br J Cancer* **65**: 552–558, 1992.
8. Thakkar NS and Potten CS, Abrogation of Adriamycin toxicity *in vivo* by cycloheximide. *Biochem Pharmacol* **43**: 1683–1691, 1992.
9. Pawlak K, Pawlak JW and Konopa J, Cytotoxic and antitumor activity of 1-nitroacridines as an aftereffect of their interstrand DNA cross-linking. *Cancer Res* **44**: 4289–4296, 1984.
10. Bertrand R, Kerrigan D, Sarang M and Pommier Y,

Cell death induced by topoisomerase inhibitors. *Biochem Pharmacol* **42**: 77–85, 1991.
11. Wyllie AH, Kerr JFR and Currie AR, Cell death: the significance of apoptosis. *Int Rev Cytol* **68**: 251–306, 1980.
12. Wasserman K, Zwelling LA, Mullins TD, Silberman LE, Andersson BS, Bakic M, Acton EM and Newman RA, Effects of 3′-deamino-3′-(3-cyano-4-morpholinyl)doxorubicin and doxorubicin on the survival, DNA integrity, and nucleolar morphology of human leukemia cells *in vitro*. *Cancer Res* **46**: 4041–4046, 1986.
13. Zwelling LA, Altschuler E, Cherif A and Farquhar D, *N*-(5,5-Diacetoxypentyl)doxorubicin: a novel anthracycline producing DNA interstrand cross-linking and rapid endonucleolytic cleavage in human leukemia cells. *Cancer Res* **51**: 6704–6707, 1991.
14. Bartkowiak D, Hemmer J and Röttinger E, Dose dependence of the cytokinetic and cytotoxic effects of epirubicin *in vitro*. *Cancer Chemother Pharmacol* **30**: 189–192, 1992.
15. Barry MA, Behnke CA and Eastman A, Activation of programmed cell death (apoptosis) by cisplatin, other anticancer drugs, toxins and hyperthermia. *Biochem Pharmacol* **40**: 2353–2362, 1990.
16. Zwelling LA, Bales E, Altschuler E and Mayes J, Circumvention of resistance by doxorubicin, but not by idarubicin, in a human leukemia cell line containing an intercalator-resistant form of topoisomerase II: evidence for a non-topoisomerase II-mediated mechanism of doxorubicin cytotoxicity. *Biochem Pharmacol* **45**: 516–520, 1993.
17. Collins RJ, Harmon BV, Souvlis T, Pope JH and Kerr JFR, Effects of cycloheximide on B-chronic lymphocytic leukaemic and normal lymphocytes *in vitro*: induction of apoptosis. *Br J Cancer* **64**: 518–522, 1991.
18. Bodley A, Liu LF, Israel M, Seshadri R, Koseki Y, Giuliani FC, Kirschenbaum S, Silber R and Potmesil M, DNA topoisomerase II-mediated interaction of doxorubicin and daunorubicin congeners with DNA. *Cancer Res* **49**: 5969–5978, 1989.
19. Del Bino G and Darzynkiewicz Z, Camptothecin, teniposide, or 4′-(9-acridinylamino)-3-methanosulfon-*m*-aniside, but not mitoxantrone or doxorubicin, induces degradation of nuclear DNA in S phase of HL-60 cells. *Cancer Res* **51**: 1165–1169, 1991.
20. Hotz MA, Del Bino G, Lassota P, Traganos F and Darzynkiewicz Z, Cytostatic and cytotoxic effects of Fostriecin on human promyelocytic HL-60 and lymphocytic MOLT-4 leukemia cells. *Cancer Res* **52**: 1530–1535, 1992.
21. Di Marco A, Zunino F and Casazza AM, Comparison of biochemical and biological methods in the evaluation of new anthracycline drugs. *Antibiot Chemother* **23**: 12–20, 1978.
22. Siegfried JM, Sartorelli AC and Tritton TR, Evidence for the lack of relationship between inhibition of nucleic acid synthesis and cytotoxicity of Adriamycin. *Cancer Biochem Biophys* **6**: 137–142, 1983.