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Biochemical Pharmacology 67 (2004) 1047–1056
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Relationship between daunorubicin concentration and apoptosis induction in leukemic cells

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Received 4 June 2003; accepted 27 October 2003

Abstract

Aiming to determine if a concentration window exists in which apoptosis induction by daunorubicin (DNR) is optimal, we studied the relationship between DNR concentration and apoptosis induction in HL60 and K562 cells and in peripheral leukemic cells isolated from three patients with acute myelogenous leukemia (AML). Cells were incubated for 2 hr with increasing DNR concentrations and thereafter for 22 hr in drug-free medium. Apoptosis was measured by detection of caspase-3-like activity and DNA fragmentation assayed by propidium iodide and flow cytometry. High DNR concentrations initiated faster apoptosis in HL60 cells and in AML cells, as shown by caspase-3 and DNA fragmentation data. DNA fragmentation into small fragments was preceded by the formation of a narrow peak on the left side of the G1 peak, most likely large DNA fragments, but further studies are required for unequivocal confirmation. This peak could easily be misinterpreted as a G1 peak without careful time monitoring. In K562 cells, no left peak was detected, apoptosis was slow and not related to concentration. In AML cells, large interindividual variations were observed in the time course of DNA fragmentation at 0.25 μg DNR/mL. In conclusion, our findings support the concept of dose intensification for optimal apoptosis induction as higher doses correlate with earlier and more rapid caspase-3 induction and DNA fragmentation in leukemic cells. The DNA fragmentation assay may be a valuable tool to determine leukemic cells' chemosensitivity to apoptosis.

Keywords: Leukemia; Apoptosis; Flow cytometry; Daunorubicin; DNA fragmentation

1. Introduction

There has been appreciable progress in the therapy of acute myelogenous leukemia (AML) over the past 20 years and this can be partially attributed to intensification of treatment, especially during remission induction. The fraction of cells killed following exposure to cytotoxic drugs usually increases with dose and several clinical studies have provided evidence that high dose chemotherapy improves treatment results [1,2]. However, in some cases intensification of dosage did not significantly improve outcome due to toxicity problems [3,4]. A therapeutic window for apoptosis induction of anticancer agents in leukemic cells has been described in studies of the induction of apoptosis by daunorubicin (DNR) or mitoxanthrone in cell lines, where an increase in drug concentration over this window resulted in less apoptosis [5–7]. This contradicts *in vitro* cytotoxicity data in which the effects correlate with dose, and has been mainly interpreted as a mechanistic switch from apoptosis to necrosis at high drug concentration. The distinction between apoptosis and necrosis is difficult and controversial. After *in vitro* incubation of leukemic cell lines with DNR, Dartsch *et al*. [8] suggested that cell death was not occurring by apoptosis but rather by necrosis due to loss of membrane integrity. Necrosis was also recently observed in Jurkat cells after a 3-day incubation with low-dose doxorubicin, an antitumoral drug closely related to DNR [9].

The coordinated regulation of cell proliferation, differentiation and death through apoptosis or necrosis is critical

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Abbreviations: CAD, caspase-activated-DNAse; CHAPS, [3-(3-cholamidopropyl)dimethylammonio-1-propanesulfonate]; DFF, DNA fragmentation factor; DISC, death inducing signalling complex; DEVD-AMC, Asp-Glu-Val-Asp-Aminomethylcoumarin; PARP, poly(ADP-ribose)polymerase; PBS, phosphate saline buffer; PI, propidium iodide; LMW, low molecular weight; NP40, nonidet p40, 4-nonylphenolpolyethylenglycol.

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doi:10.1016/j.bcp.2003.10.025

for normal tissue development. In a wide range of cultured tumor cells, apoptosis initiation is an important event for the cytotoxic effects of anticancer agents [10]. Tumor cell chemosensitivity may be related to the apoptotic functional capacity of the cells and apoptosis determination has been proposed as a new approach to evaluate chemosensitivity [11–13].

During the apoptotic process, proteolysis is mediated by the caspases, a group of cysteine proteases. Among these proteases, the DEVD (asp-glu-val-asp) cleaving caspases have been shown to play a critical role in the initiation of apoptosis and to cleave poly(ADP-ribose)polymerase (PARP) early during apoptosis. Caspase-3 is one important component of the caspase cascade, and is activated by two sequential proteolytic events leading to rapid and irreversible proteolysis and DNA fragmentation through caspase-activated-DNAse (CAD, also known as DFF, DNA fragmentation factor) activation. It is an essential protease in the process of apoptosis since it is implicated in the DNA fragmentation process. It is activated in the extrinsic pathway through drug-induced formation of a FADD- and caspase-8-containing CD95 death inducing signalling complex (DISC). Caspase-3 is also involved in the intrinsic pathway through the formation of the apoptosome (Apaf-1 cytochrome *c* complex). This leads to the activation of caspase-9 that subsequently activates caspase-3 [14].

In the present study, we have investigated *in vitro* the effects of increasing DNR doses on the induction of apoptosis in leukemic cells in order to determine if a concentration window exists where apoptosis induction is optimal. To mimic the clinical situation two leukemic cell lines and mononuclear cells isolated from three patients with AML were pulse incubated for 2 hr with various concentrations of DNR and thereafter incubated in drug-free medium. We measured the type II ''effector'' caspase or caspase-3-like activity (including caspase-3 and -7) as early apoptosis marker. Late apoptosis was determined from DNA fragmentation by propidium iodide (PI) staining.

2. Materials and methods

2.1. Cell lines and incubation procedure

We used the human promyelocytic leukemia cell line HL60 and the erythroleukemia cell line K562, obtained from American Type Culture Collection. Cell lines were maintained in a humidified, 5% CO_2 incubator at 37° in RPMI 1640 cell culture medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL) and subcultured twice a week. For time course analyses of apoptosis, cells at a concentration of 10^6 mL^{-1}, were incubated for 2 hr with increasing concentrations of DNR (0, 0.25, 0.5, 1, 2, 4 μg/mL; RPR-Bellon). Thereafter the cells were washed twice with ice cold phosphate-buffered saline (PBS) by centrifugation at 550 g for 5 min at 4° and resuspended in drug-free medium at the same cell concentration. Cells were harvested at different time points and washed twice with ice cold PBS before the determination of cell number using a Coulter counter Z2 (Beckman Coulter), caspase-3-like activity, and DNA fragmentation as described below.

2.2. Clinical samples

Heparinized peripheral blood samples were obtained from three patients with AML. Mononuclear cells were isolated from 5 mL blood by centrifugation on 3 mL Lymphoprep (Nycomed) at 550 g for 15 min at 4°. After two washes with PBS, the cell number was determined and incubation was carried out directly with 1 million cells/mL following the protocol described for cell lines. The study was approved by the ethics committee at Karolinska Hospital.

2.3. In vitro caspase assay

The measurement of caspase-3-like activity was performed using DEVD-AMC as substrate (Peptide Institute). Proteolytic cleavage of the 7-amino-methylcoumarin-derived substrate DEVD-AMC gives a bright blue fluorescent product. The measurements were performed in triplicate in black 96-well microplates with 1 million cells using a modified fluorometric assay [15]. Briefly, the reaction was initiated by the addition of a reaction buffer composed of 100 mM HEPES, 10% sucrose, 5 mM dithiothreitol (DTT), 0.0001% NP40, and 0.1% CHAPS pH 7.25, all purchased from Sigma-Aldrich. The fluorescence was measured after 30 min at 37° (λ_{exc}: 340–360 nm, λ_{em}: 440–460 nm, sensitivity, 100) using a FL600 reader (Bio-Tek) and expressed in milliunits of fluorescence per million cells.

2.4. DNA fragmentation with PI staining and flow cytometry

We used a propidium iodide-based staining procedure as previously described [16]. The appearance of cells less intensively stained than G1 cells (sub-G1 or A_o cells) in flow cytometric DNA histograms was used as a marker of apoptosis. Briefly, a cell pellet containing 1×10^6 cells was gently resuspended in 0.5 mL PI staining solution (PI 50 μg/mL in 0.1% (w/v) sodium citrate plus 0.1% (v/v) Triton X-100; Sigma-Aldrich Co.) in 12 mm × 75 mm polypropylene tubes (Becton Dickinson). The tubes were placed at 4° in the dark for 20 min to 3 days before flow cytometry measurements. The PI fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton and Dickinson, Mountain View) with a single 488-nm Argon laser. The red fluorescence due to PI staining of DNA was detected in the FL-3 channel and the data were

registered on logarithmic and linear scales. The forward scatter (FSC) and side scatter (SSC) of particles were simultaneously measured. The flow rate was set at 12 μL/s and at least 10,000 target events were collected for each sample. Analysis was performed using Cell Quest™ software.

3. Results

3.1. DNR induction of apoptosis in HL60 cells

The time course of caspase-3-like activity after a 2-hr pulse incubation of HL60 cells with increasing DNR concentrations is presented in Fig. 1. With the highest DNR concentration caspase activity increased, peaked rapidly and thereafter decreased with time. At lower DNR concentrations caspase-3 activity appeared later and reached lower levels, clearly illustrating that a study of caspase activity at one time point would be misleading.

As a second apoptosis detection method we determined DNA fragmentation by analyzing PI staining with flow cytometry. Here again, a time course analysis at increasing concentrations gave a more correct picture of DNA fragmentation, as illustrated in Fig. 2. DNA fragmentation was already observed after 2 hr at a concentration of 1 μg/mL or more, but the hypodiploid population mainly appeared as a narrow peak on the left side of the G0/G1 peak, and increased with time. After 4 hr, this "left peak" was observed at all concentrations tested. It became higher and narrower with time, which is particularly well illustrated for 2 μg DNR/mL after 7.5 and 22 hr. At 4 μg DNR/mL, the G1 peak was already quite reduced after 4 hr and the left peak became so narrow that it could erroneously be assigned as a G1 peak without a time observation. After 22 hr, a peak of small DNA fragments was also detected, mostly in cells incubated with 2 and 4 μg DNR/mL.

By assuming that the low molecular weight (LMW) DNA and these "left peak" fragments represented the apoptotic population, we obtained a time course of apoptotic cells in HL60 cells with increasing DNR concentrations, as shown in Fig. 3. It was similar to the time profile of caspase activity, with a rapid apoptosis induction at high DNR concentration, already culminating after 4 hr with more than 90% apoptotic cells, and a delayed apoptosis induction at lower DNR concentrations.

3.2. DNR induction of apoptosis in K562 cells

In K562 cells, as in HL60 cells, caspase-3-like activity occurred earlier and also with higher peak activities with increasing DNR concentrations, but this was observed at much higher DNR concentrations (4 and 8 μg/mL) (data not included). Moreover, the apoptotic picture differed markedly from that observed in HL60 cells; caspase-3 activity being detected later, culminating after 8 hr for the highest concentrations (8 μg/mL) and much later for the lower concentrations (40 hr for 1 μg/mL). In comparison with HL60 cells, in which apoptosis was almost totally complete after 22 hr, DNA fragmentation was barely detectable in K562 cells before this time. At low DNR concentrations K562 cells were mainly arrested in G2/M and at high drug concentrations the block was observed in G0/G1. No left peak was detected close to G0/G1. After 22 hr, a small peak of LMW DNA fragments was detected, increasing slowly at all concentrations to culminate at 54%

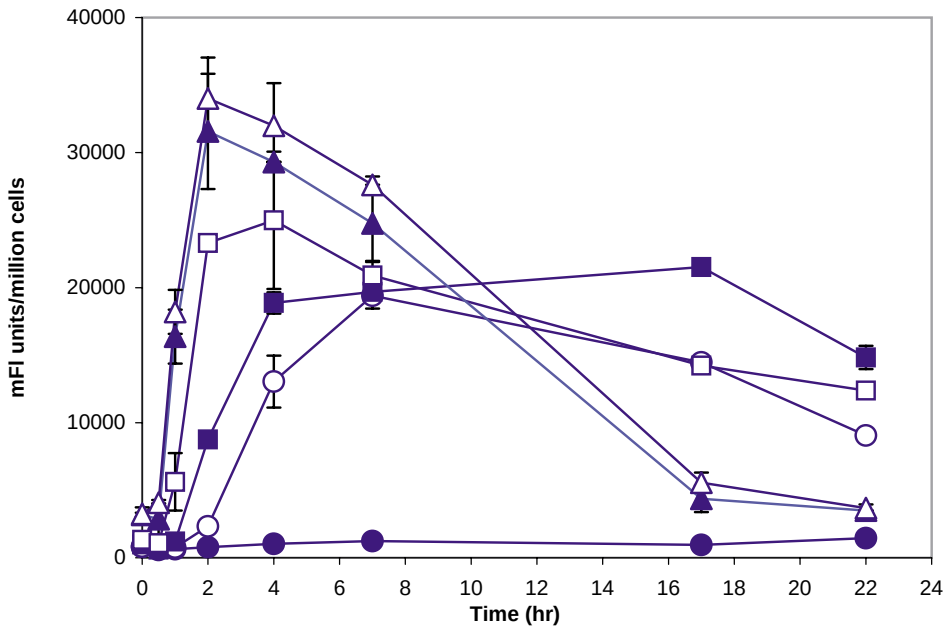

Fig. 1. Time course of caspase-3 activity after a 2-hr pulse incubation of HL60 cells at 1 million cells/mL with DNR at concentrations: 0 (●), 0.25 (○), 0.5 (■), 1 (□), 2 (▲), 4 (△) μg/mL. Mean and standard deviation of triplicate determinations.

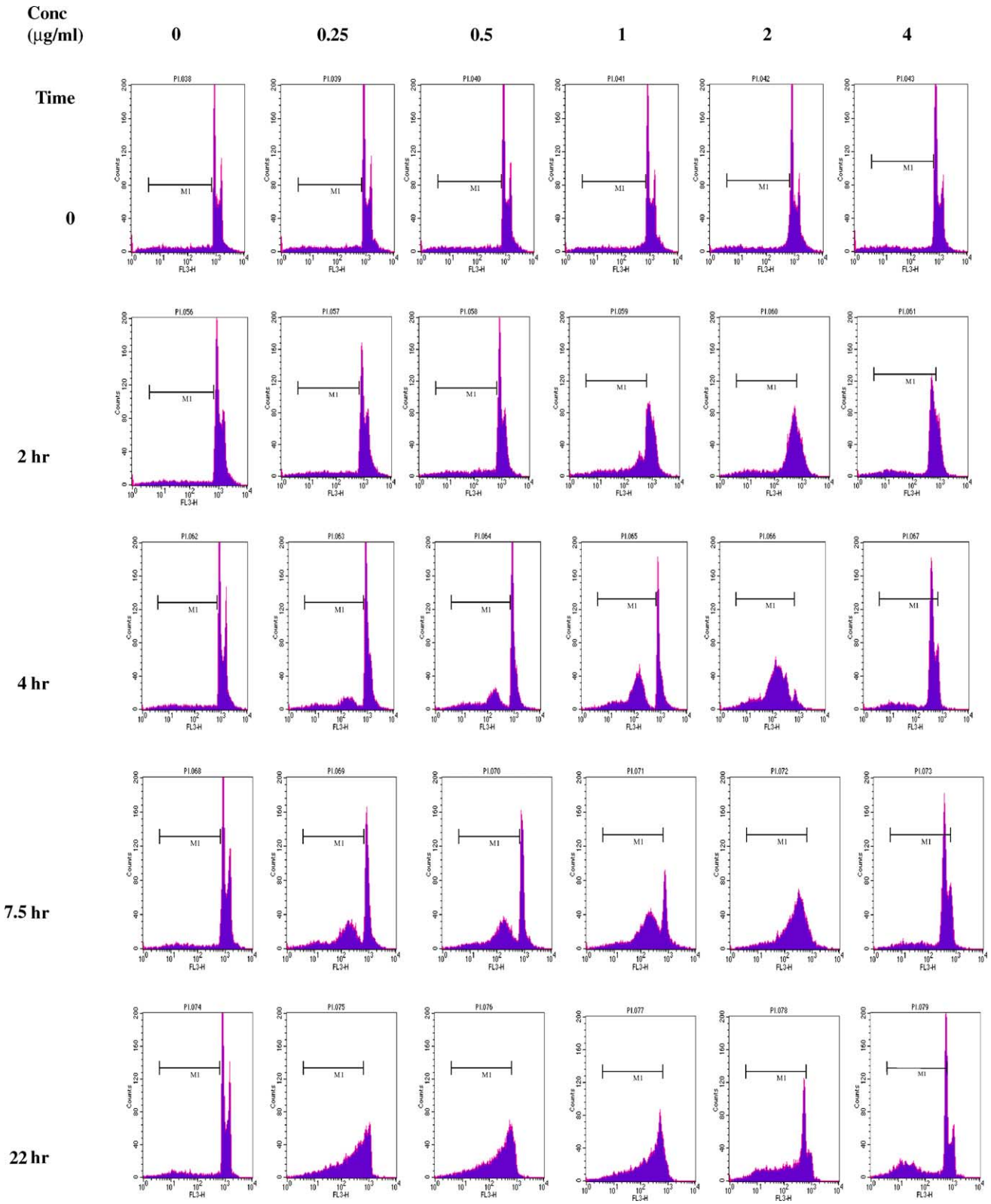

Fig. 2. Flow cytometry diagrams illustrating the time course of PI staining in HL60 cells after a 2-hr pulse incubation with increasing DNR concentrations.

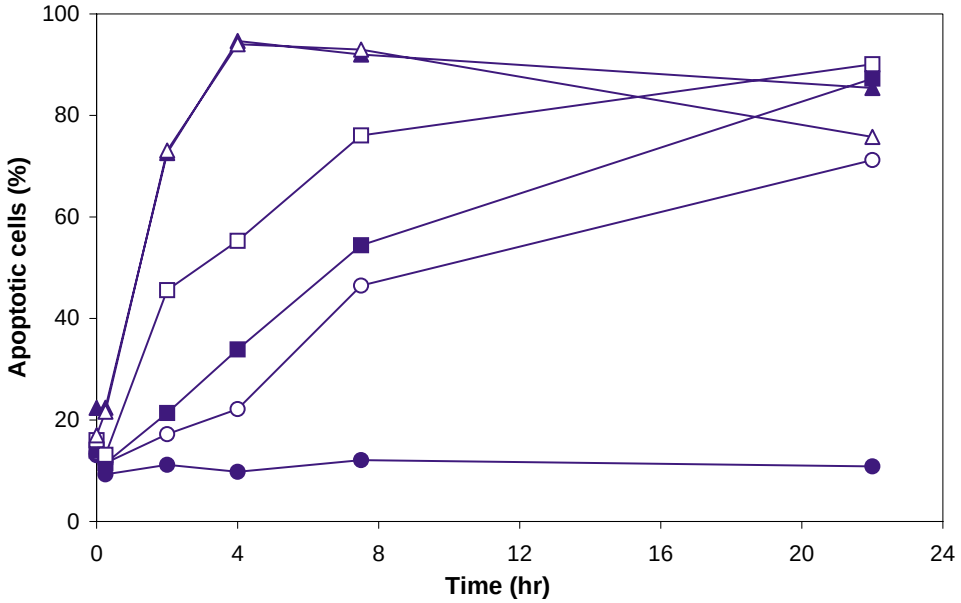

Fig. 3. Time course of DNA fragmentation after a 2-hr pulse incubation of HL60 cells at 1 million cells/mL with DNR at concentrations: 0 (●), 0.25 (○), 0.5 (■), 1 (□), 2 (▲), 4 (△) μg/mL.

at 1 μg DNR/mL after 63 hr. The fraction of apoptotic cells did not increase further with higher DNR concentrations.

3.3. DNR induction of apoptosis in leukemic cells from AML patients

DNR induction of apoptosis was also studied in isolated mononuclear blood cells from three patients with AML. The time course of caspase-3-like activity with increasing DNR concentrations was determined in leukemic cells isolated from one patient and had a similar pattern to that observed in HL60 cells, with a delayed response at low DNR concentrations (data not included). In the flow cytometry diagrams of Fig. 4, presenting the time course of DNA PI staining, we observed that for the three highest DNR concentrations the hypodiploid fraction was detected after 6 hr, mainly as a narrow "left peak" and occurred 2 hr after maximal caspase-3 activity. After 10 hr, the "left peak" peak was also detected in the cells incubated with the lower concentrations, 0.5 and 0.25 μg/mL. After 24 hr, some LMW DNA fragments appeared in the cells incubated with 2 and 4 μg/mL and after 46 hr LMW fragments increased in all treated cells. The presence of a "left peak" is better illustrated in Fig. 5, showing the flow cytometry diagrams obtained when the data were collected with a linear amplifier. After a 10-hr incubation in drug-free medium, the high G1 peak observed in the controls was progressively replaced by this "left peak" with increasing DNR concentrations. After 46 hr, this peak was reduced at high DNR concentrations and replaced by a LMW DNA fragment peak observed in the FL3 channel between 0 and 100. The time course of apoptotic cells (sum of LMW DNA fragments and "left peak") of these cells with increasing concentrations displayed a profile similar to HL60 cells, with a delayed fragmentation at lower DNR concentrations as illustrated in Fig. 6. The interindividual variation in the time courses of DNR induction of apoptosis at 0.25 μg/in leukemic cells from three patients with AML is presented in Fig. 7.

4. Discussion

In order to investigate the eventual existence of a therapeutic window for apoptosis induction, this study examined the influence of DNR concentrations on apoptosis induction in two leukemic cell lines and leukemic cells isolated from patients with AML. We determined caspase-3-like activity and DNA fragmentation at concentrations between 0.25 and 4 μg/mL (0.44 and 7 μM), which covers and exceeds the range of the peak concentration in plasma after a bolus injection. At a dose of 45 mg/m^2, peak plasma levels between 0.25 and 0.5 μg/mL (0.45 and 0.9 μM) were found [17,18]. The data presented here demonstrated (i) that the highest DNR concentrations studied did not inhibit caspase-3 activation and DNA fragmentation but rather induced a quicker response than the lower concentrations; (ii) in HL60 cells and leukemic cells isolated from AML patients, DNA fragmentation was a two step process; and (iii) that isolated leukemic cells displayed large interindividual differences in the time course and extent of their DNA fragmentation induced by DNR.

Our data do not support the existence of a concentration window for apoptosis induction in leukemic cells by DNR, if caspase-3-like activity and DNA degradation reflects apoptosis. Indeed, caspase-3 activation occurred faster and earlier at high DNR concentrations and there was good

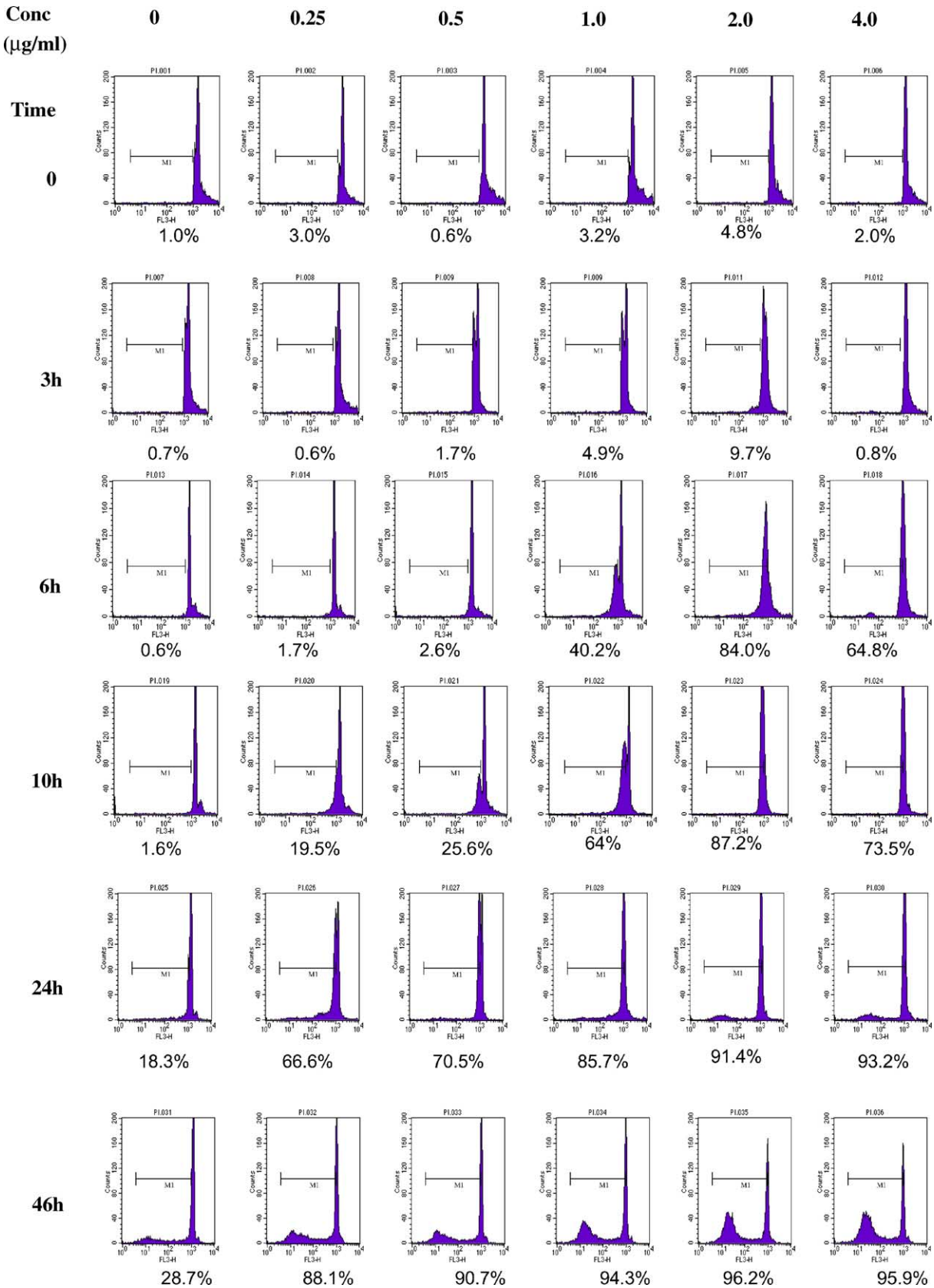

Fig. 4. Flow cytometry diagrams illustrating the time course of PI staining of mononuclear cells isolated from an AML patient after a 2-hr incubation at 1 million cells/mL with increasing DNR concentrations.

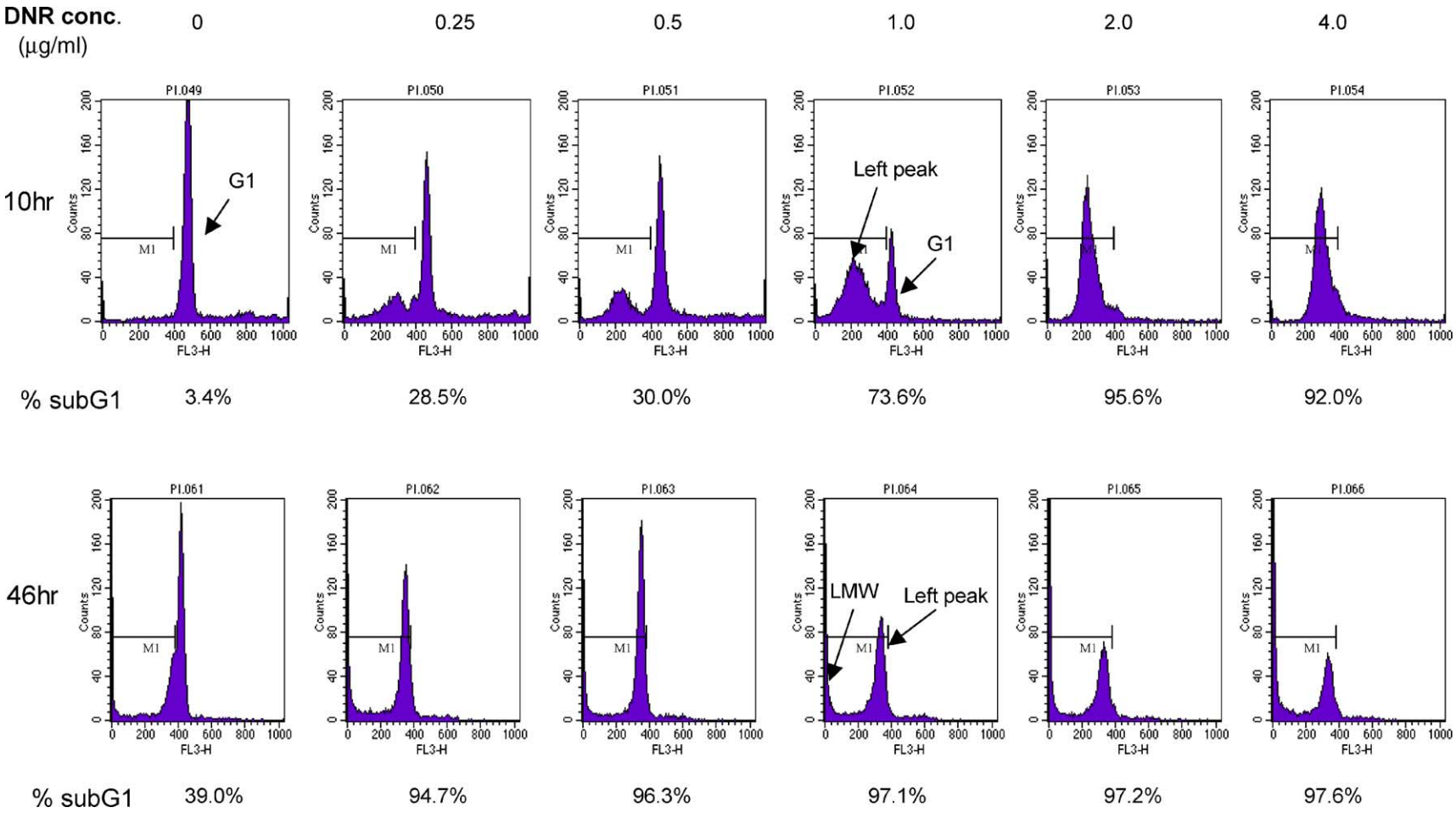

Fig. 5. Flow cytometry diagrams (obtained with linear data collection) of PI staining in mononuclear cells isolated from an AML patient, 10 or 46 hr after a 2-hr incubation at 1 million cells/mL with increasing DNR concentrations.

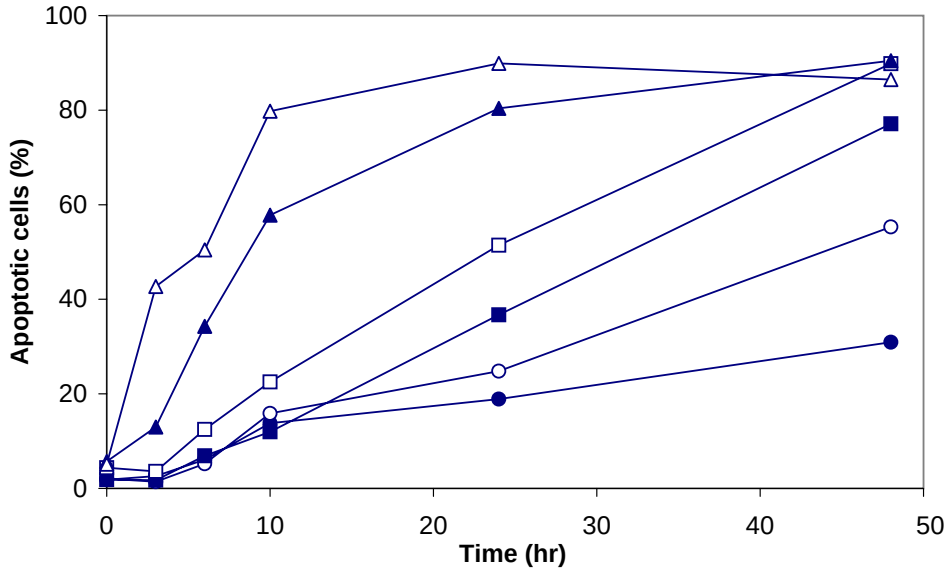

Fig. 6. Time course of DNA fragmentation of mononuclear cells isolated from an AML patient, after a 2-hr pulse incubation at 1 million cells/mL with DNR at concentrations: 0 (●), 0.25 (○), 0.5 (■), 1 (□), 2 (▲), 4 (△) μg/mL.

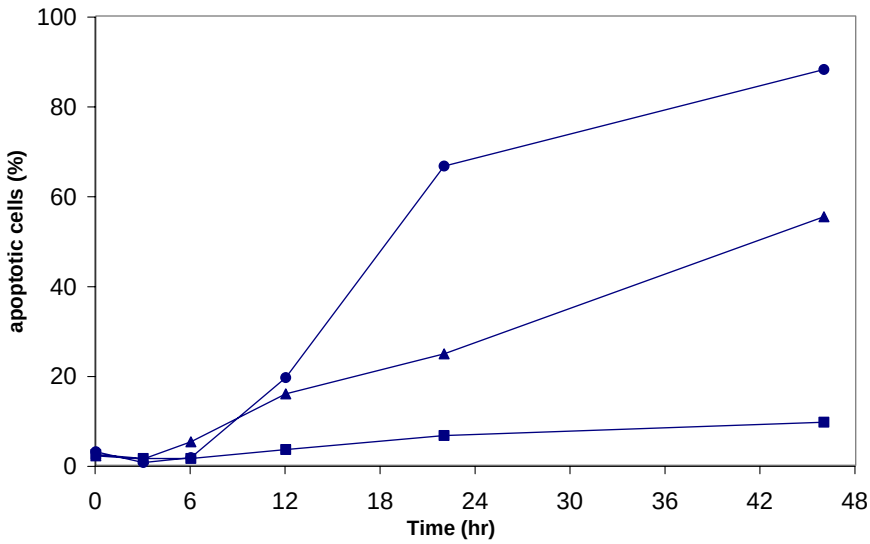

Fig. 7. Time course of DNA fragmentation after a 2-hr pulse incubation, with 0.25 μg/mL DNR and at 1 million cells/mL, of mononuclear cells isolated from three patients with AML: A (●), B (■), C (▲).

agreement between caspase-3 activity and extent of apoptosis measured as DNA fragmentation. In K562 cells, however, the highest DNR concentrations did not induce a faster DNA fragmentation. The resistance of K562 cells to apoptosis, due to a deficiency of Apaf-1 protein and the absence of Bax translocation to mitochondria, has been previously described [6,19,20].

Efferth *et al.* [5] reported that apoptosis detected with DNA PI staining was saturable as a function of drug concentration in the medium and decreased at the highest concentrations after exposure of sensitive, resistant cell lines, and leukemic cells from six patients with AML to DNR. In contrast to our study, they incubated cells continuously for 6 hr or more with DNR concentrations between 0.1 and 10 μg/mL. The decline in apoptosis at high drug concentrations was explained by an *in vitro* mechanistic switch from the apoptotic to the necrotic death mode. By incubating KG1 cells with 5 or 10 μg/mL DNR (8.8 and 17.7 μM) for 48 hr, a significant increase in necrosis was detected by 7-amino-actinomycin D staining. Under our experimental conditions, the initial cell death mode can be explained by apoptosis induction by DNR at all concentrations tested, but the slight decrease in DNA fragmentation observed after 22 hr in HL60 cells treated with 2 or 4 μg DNR/mL is probably due to secondary necrosis after apoptosis. Another group, using lower DNR concentrations (up to 169 ng/mL, 300 nM), did not observe a concentration-related apoptosis decrease after a 16-hr

continuous incubation of U937 cells [7]. However, a reduction of apoptosis at higher drug concentrations was observed in this cell line after a16-hr incubation with mitoxanthrone at 0–300 nM.

The morphological definition of apoptosis and necrosis is based on membrane integrity; cells stained with propidium iodide in isotonic buffer are assumed to be necrotic as PI is unable to cross intact cell membranes. In a recent study of cell death induction by DNR (200 nM during 6 hr) in HL60 cells and Jurkat cells it was suggested that the mode of cell death was necrosis and not apoptosis [8]. These authors observed that, after staining with PI, the cells with condensed chromatin also displayed red fluorescence which was interpreted as a loss of membrane integrity.

We did not succeed in our attempts to measure necrosis induced by DNR, as the fluorescence spectra of propidium iodide and daunorubicin are fairly close and we could not discriminate between the contributions of DNR and PI in the red fluorescence observed in the cells stained with PI. The fluorescence characteristics of DNR and its high cellular uptake make PI a nonreliable marker of membrane integrity. Using the same method, Sugimoto *et al*. [9] detected necrosis induction in Jurkat cells after a 60-hr incubation with a low dose of doxorubicin (40 nM), an anthracycline closely related to DNR, and also with AraC and aclarubicin. However, at drug concentrations of 200 nM cell death occurred by apoptosis. The precise transition between apoptosis and necrosis remains controversal. Anthracyclines are known to produce reactive oxygen species (ROS) which have been implicated in apoptosis induction at low drug levels but which trigger necrosis at higher levels. A dual regulation of caspase activity has also been described in Jurkat cells treated with hydrogen peroxide, a typical ROS generator [21,22]. At high concentrations of hydrogen peroxide, caspase activity disappeared and the cells died by necrosis due to an inactivation of the cysteine-dependent caspases. Another study observed that high DNR concentrations caused a sharp ATP depletion that is characteristic of necrotic cell death in K562 and CEM cells [6].

In this study, we also observed that DNA fragmentation into LMW fragments was preceded by the appearance of a narrow peak (on the left side of the G1 peak) in HL60 cells and leukemic cells isolated from AML patients. The position of this peak, close to the 2N DNA population and its transformation into a wider peak of LMW DNA fragments support the hypothesis that it most likely represents DNA large fragments. DNA fragmentation at the internucleosomal linker regions, a process called internucleosomal cleavage, is a characteristic of apoptosis and produces ladders of fragments of 180–200 bp that are easily identified by agarose gel electrophoresis. Some authors suggest that intranucleosomal cleavage of DNA into high MW fragments (50–300 kb) represents major damage that cannot be repaired and may contribute to an amplification phase of apoptosis [23,24]. Those studies showed that the generation of such fragments requires CAD endonuclease activity. Given that caspase-3 mediates the activation of CAD, its activation is required to generate the 50–300-kb DNA fragments [23]. This has been reported by Sakahira *et al*. [25] in Jurkat cells, in which the expression of a caspase-3 resistant mutant of ICAD blocked the generation of 50-kb DNA fragments in response to taurosporine. In our kinetic study of DNA degradation, the inclusion of the left peak in the hypodiploid fraction, representing DNA degradation, resulted in a higher apoptosis index for the highest DNR concentrations in all cells studied, with the exception of K562 cells, and the curves obtained correlated very well with the kinetics of caspase-3-like activity in the same cells, although with a short delay. We did not detect this left peak in K562 cells, in agreement with the results of Huang *et al*. [24] who demonstrated an inhibition of high MW DNA fragmentation activity in CEM cell extracts by K562 cell extracts. The high MW DNA fragments are not visible by classical agarose gel electrophoresis, but can be detected by pulsed-field gel electrophoresis [26,27]. Badran *et al*. [28] used this method to monitor an *in vivo* apoptotic response in circulating leukemic cells from a patient with chronic lymphocytic leukemia (CLL) treated with fludarabine while conventional cytometric parameters of apoptosis remained negative. They claimed that this assay is a sensitive method for monitoring *in vivo* responses to chemotherapy. The use of flow cytometry to measure DNA degradation by PI staining and detect high MW DNA fragments could be a very interesting method to evaluate as an alternative to pulsed-field gel electrophoresis.

In conclusion, a 2-hr pulse incubation with DNR at concentrations higher than 0.44 μM induced apoptosis in HL60 cells and isolated AML cells and the fastest effect was obtained with the highest concentration tested (7 μM) through rapid degradation of DNA. The use of PI staining and flow cytometry to monitor *in vitro* and *in vivo* response to chemotherapy by detection of low and high MW DNA fragments is attractive but it warrants further studies like a direct comparison with pulsed-field gel electrophoresis. Some isolated AML cells were quite resistant to apoptosis induction and the large interindividual variation observed in the time course of DNA fragmentation for the isolated AML cells is interesting and could reflect variations in sensitivity *in vivo*. Further studies are needed to elucidate if apoptosis induction *in vitro* by DNR in leukemic cells could predict treatment outcome in the clinic.

Acknowledgments

This work was supported by the Karolinska Institute, and the China Scholarship Council. We thank Associate Prof. R.A. Harris for linguistic advice.

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