Anthraquinone antitumour agents, doxorubicin, pirarubicin and benzoperimidine BP1, trigger caspase-3/caspase-8-dependent apoptosis of leukaemia sensitive HL60 and resistant HL60/VINC and HL60/DOX cells

Robert Nowak and Jolanta Tarasiuk

We examined the effect of selected anthraquinone antitumour agents – doxorubicin (DOX), pirarubicin (PIRA) and benzoperimidine BP1 – on inducing apoptosis of the sensitive leukaemia HL60 cell line and its multidrug resistance sublines overexpressing P-glycoprotein (HL60/VINC) and multidrug resistance-associated protein 1 (HL60/DOX). All agents used at IC_{50} and IC_{90} were able to influence the cell cycle of sensitive HL60 and resistant cells and induce apoptosis. Interestingly, it was seen that HL60/VINC cells were more susceptible to undergo caspase-3/caspase-8-dependent apoptosis induced by the studied anthraquinone compounds compared with HL60 and HL60/DOX cells. However, the examined agents did not change the expression of Fas receptors on the surface of HL60-sensitive and-resistant cells. *Anti-Cancer Drugs* 23:380–392 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The multidrug resistance (MDR) of tumour cells to a wide array of antitumour drugs, which are structurally diverse and have different mechanisms of action, constitutes the major obstacle to the successful treatment of cancer [1,2]. The MDR phenomenon is very frequently associated with the presence of membrane transporters belonging to the ATP-binding cassette protein family [P-glycoprotein (P-gp), multidrug resistance-associated protein 1 (MRP1), breast cancer resistance protein/mitoxantrone resistance protein 1]. These transporters are responsible for the active ATP-dependent efflux of drugs out of resistant cells, resulting in the decreased intracellular accumulation insufficient to inhibit resistant cell proliferation [3].

It is proposed that, in addition to mediating drug resistance through drug efflux, MDR pumps could inhibit caspase-dependent apoptosis of resistant cells [4,5]; however, they remain ineffective in protecting against caspase-independent cell death [6–8]. It has also been suggested that the inhibition of apoptosis by MDR pumps could be related to the decrease in ATP level, inhibiting procaspase-8 activation (the extrinsic pathway) and apoptosome formation necessary for procaspase-3 activation (the intrinsic mitochondrial pathway) [4,6]. There are also some reports suggesting that the protection of resistant cells against apoptotic stimuli by MDR pumps could be linked to the cytoplasm alkalization disturbing caspase activation [4,9]. In addition, some reports show that it may be related to the flip–flop transport of membrane lipids by these proteins causing changes in the membrane lipid distribution and as a consequence disturbing the activation of death receptors (crucial for the induction of the extrinsic apoptotic pathway) and synthesis of ceramide (second activator of caspases) [10–12].

However, some recently published data showed that the overexpression of P-gp and MRP1 in promyelocytic leukaemia cells did not change the caspase-3-dependent apoptotic pathway induced by curcumin and ultraviolet-C (UVC) irradiation [13]. It was also reported that these MDR pumps did not confer resistance to Fas-induced apoptosis and caspase-3 activation in a large series of P-gp-expressing and MRP-expressing drug-resistant leukaemia sublines [14]. Moreover, there are some reports showing that cells overexpressing P-gp are surprisingly more sensitive to apoptosis stimuli acting through the mitochondrial and death receptor-mediated apoptotic pathways [15,16]. Further, caspase-3-dependent cleavage of P-gp during apoptosis of T-lymphoblastoid CEM cells in response to various inducers was also recently observed. On the basis of these findings, it was proposed that caspase-3 cleavage of P-gp would be a proapoptotic self-enhancing mechanism favouring the apoptosis of tumour cells overexpressing P-gp [17].

Thus, taking into account the diversity of apoptotic stimuli and the complexity of apoptotic pathways in different tumour cells, it can be stated that our knowledge about the mechanisms responsible for the inhibition

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of apoptosis in MDR cells necessary for the development of more effective therapy strategies is far from satisfactory. Therefore, further systematic studies using various antitumour agents and MDR cells are required to identify the crucial apoptotic factors in these cells to be targeted by new chemotherapeutics.

To address this issue, we examined in a comparative manner the effect of three anthraquinone antitumour agents – doxorubicin (DOX), pirarubicin (PIRA) and benzoperimidine BP1 (Fig. 1) – in inducing apoptosis of the human promyelocytic sensitive leukaemia HL60 cell line and its MDR sublines exhibiting two different phenotypes of MDR related to the overexpression of P-gp (HL60/VINC) or MRP1 (HL60/DOX).

Materials and methods

Chemicals

DOX and PIRA were kindly provided by Pharmacia-Upjohn (Milano, Italy). Purified benzoperimidine BP1 was prepared according to the procedure outlined in Stefańska *et al*. [18].

Ribonuclease A and propidium iodide were purchased from Sigma-Aldrich (Saint Louis, Missouri, USA), proteinase K from Merck (Darmstadt, Germany) and DNA size markers (100–1000 bp) from AppliChem (Darmstad, Germany). Potassium hydroxide and perchloric acid were obtained from POCh (Gliwice, Poland).

High-performance liquid chromatography (HPLC)-grade reagents acetonitrile (Merck), potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, potassium chloride (Fluka, Buchs, Switzerland) and ATP standards (Sigma-Aldrich) were used.

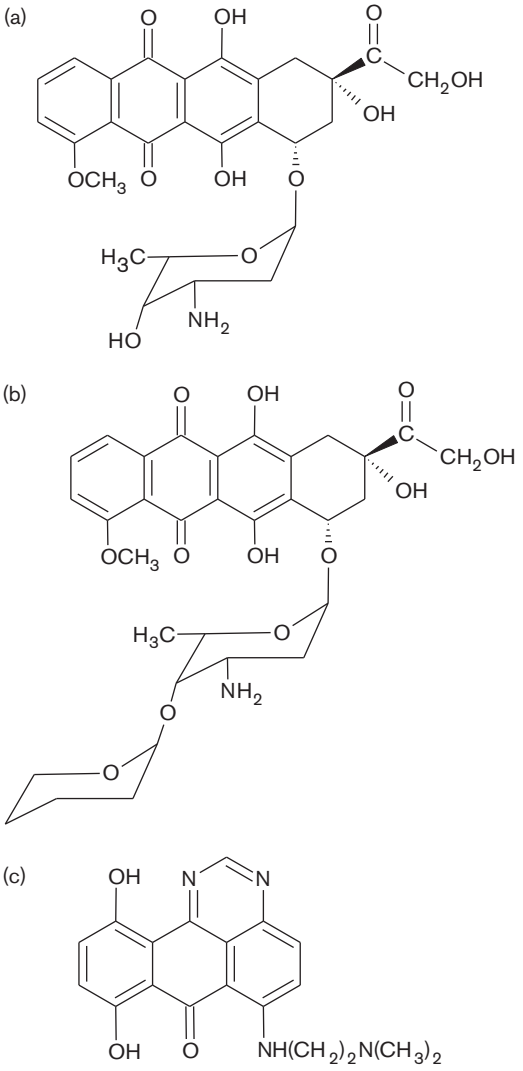
Cell culture

The HL60 human promyelocytic leukaemia cell line (Division of Biology, Kansas State University, Manhattan, Kansas, USA) and its resistant sublines HL60/VINC (overexpressing P-gp) [19] and HL60/DOX (overexpressing MRP1) [20,21] were cultured. The cells were grown in RPMI 1640 (Gibco Limited, Grand Island, New York, USA) medium supplemented with 2 mmol/l glutamine and 10% fetal bovine serum (Gibco Limited) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. HL60/VINC cells were cultured in the presence of 10 nmol/l vincristine, and HL60/DOX cells were cultured in the presence of 200 nmol/l DOX. All cultures (HL60, HL60/VINC and HL60/DOX) were initiated at a density of 10^5 or 5×10^5 cells/ml and grew for 72 or 24 h for steady-state or logarithmic phase of growth, respectively. Cell viability was assessed using trypan blue exclusion.

Cytotoxicity assays

For each cell line, the cytotoxic effects of DOX, PIRA and BP1 were determined by incubating cells (10^4 cells per well) with 12 different concentrations of these compounds for 72 h in standard 96-well plates. The concentrations of the tested compounds required to inhibit cell growth by 50% (IC_{50}) or 90% (IC_{90}) were determined by counting viable cells in the presence of trypan blue using a Burker hemocytometer. For calculating the percentage of cell growth inhibition, untreated cell samples maintained in culture medium for 72 h were used as the control.

Fig. 1



Structures of examined anthraquinone compounds: (a) doxorubicin (DOX), (b) pirarubicin (PIRA) and (c) benzoperimidine BP1.

Intracellular accumulation of examined anthraquinone compounds

The cells in logarithmic growth phase were suspended in 1 ml of 20 mmol/l HEPES buffer containing 132 mmol/l NaCl, 3.5 mmol/l KCl, 1 mmol/l $CaCl_2$, 0.5 mmol/l $MgCl_2$ and 5 mmol/l glucose (pH 7.25) at 37°C to a final concentration of 10^6 cells/ml. At t_0, 10 µl of the stock

solution was added to this suspension yielding a concentration of 1 μmol/l, which was then incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 15 min in the case of PIRA and BP1 and 5 h in the case of DOX. After the indicated incubation time, the intensity of the fluorescence signal was measured by flow cytometry (FACSCalibur, Becton Dickinson, Franklin Lakes, New Jersey, USA). The measurements were taken within the FL-2 fluorescence channel (bandpass filter, $\lambda = 585/42$ nm) for DOX and PIRA and within the FL-3 fluorescence channel (longpass filter, $\lambda \geq 670$ nm) for BP1, after excitation with an argon-ion laser ($\lambda_{ex} =$ 488 nm). For each experimental point, a fluorescence signal of 10^4 events was measured. The data were analysed using BD CellQuest Pro (Becton Dickinson) and WinMDI (ver. 2.8) software (*http://www.methods.info/software/flow/winmdi.html*).

Cell cycle distribution analysis using flow cytometry

The cells (5×10^5/ml) incubated in the presence of examined anthraquinone compounds (DOX, PIRA, BP1; at both IC_{50} and IC_{90}) were collected at indicated incubation time points by centrifugation (300*g*, 5 min, 4°C) and washed twice with 1 ml of ice-cold PBS. The obtained cell pellet was suspended in 0.5 ml PBS and fixed in 3 ml of ice-cold 70% ethanol at −20°C. The fixed cells were centrifuged (300*g*, 5 min, 4°C), the pellet was rehydrated in PBS and then centrifuged again. The cells were incubated with 0.5 ml PBS containing RNase A (100 μg/ml) and propidium iodide (20 μg/ml) in the dark for 0.5 h at room temperature. Samples were analysed by flow cytometry (FACScan, Becton Dickinson). The red fluorescence (FL-2) of 10^4 single events was recorded using a laser beam at 585/42 nm ($\lambda_{ex} = 488$ nm) to measure the DNA content. The percentage of cells in each phase of the cell cycle and the percentage of apoptotic cells (subdiploid DNA region) were calculated using WinMDI 2.8 software.

DNA fragmentation analysis

The cells were incubated for up to 96 h with DOX, PIRA and BP1 (IC_{90}). After the indicated incubation times, DNA fragmentation analysis was carried out according to the method described by Składanowski [22]. Briefly, the cells (1.5×10^6) were washed with ice-cold PBS and treated at 50°C for 1 h in lysis buffer (10 mmol/l TRIS-HCl, 10 mmol/l EDTA, 10 mmol/l NaCl, 0.5% SDS, pH 7.4) containing 0.5 mg/ml proteinase K. Thereafter, 1 mg/ml ribonuclease A was added and the samples were incubated for 2 h at 50°C. Then, 1 mol/l NaCl was added and the samples were centrifuged for 30 min (500*g*; 4°C). Supernatants were collected and DNA was precipitated in 96% ethanol overnight at −20°C. The obtained DNA samples were dissolved in a buffer containing 10 mmol/l TRIS-HCl, 1 mmol/l EDTA and 15 mmol/l NaCl (pH 7.4) and separated by electrophoresis in 1.8% agarose gel containing 0.5 μg/ml ethidium bromide. The DNA samples were visualized and photographed under UV illumination.

Caspase-3 and caspase-8 activity assays

The activity of caspase-3 and caspase-8 in the examined cells was measured using commercial colorimetric kits (Sigma-Aldrich). After the indicated incubation times, cells (10^6) were centrifuged (300*g*, 5 min, 4°C), washed with 1 ml PBS and again centrifuged under the same conditions. Lysis buffer was added to the cell pellet and samples were incubated for 20 min on ice. Thereafter, they were centrifuged (20 000*g*, 10 min, 4°C) and the supernatant was incubated for 12 h at 37°C in the presence of caspase-3 (Ac-DEVD-pNA) or caspase-8 (Ac-IETD-pNA) substrate. The activity of caspase-3 and caspase-8 was determined by absorption measurements of product concentrations carried out at 405 nm using a UVM 340 microplate reader (ASYS Hitech, Eugendorf, Austria).

Determination of intracellular ATP levels

The sample containing 10^6 cells cultured for 24 (logarithmic phase of growth) or 72 h (steady-state phase of growth) was centrifuged (300*g*, 5 min, 4°C). After removal of the supernatant, the pellet was washed with 1 ml of ice-cold PBS and the sample was centrifuged again under the same conditions. The washed cells were resuspended in 200 μl of PBS and maintained at −20°C for 20 min. Thereafter, the sample was thawed and 200 μl of 1.3 mol/l $HClO_4$ was added. The obtained mixture was centrifuged at 19000*g* for 10 min at 4°C. The supernatant (300 μl) was neutralized with 2 mol/l KOH solution and centrifuged (19000*g*, 10 min, 4°C) again. The collected supernatant was frozen until HPLC analysis. ATP concentrations were determined using the HPLC method [23]. Aliquots of 20 μl of the obtained samples were injected onto a chromatographic column and ATP was separated using a linear phosphate buffer gradient elution system [buffer A: 150 mmol/l KH_2PO_4, 150 mmol/l KCl adjusted to pH 6.0 with K_2HPO_4; buffer B: 15% (v/v) solution of acetonitrile in buffer A] at a flow rate of 0.666 ml/min. Peaks were detected by absorption measurements at 254 nm. The cycle time was 12.8 min between injections. The analytic column was maintained at a constant temperature of 20.5°C. Sample peaks were integrated, calibrated and quantified using an HPLC two-dimensional chromatography data system operating on Chemstation Software for Windows 98 (Hewlett Packard, Waldbronn, Germany).

Fas expression analysis

Fas expression was analysed using fluorescein isothiocyanate-conjugated anti-Fas monoclonal antibodies (Becton Dickinson) according to the manufacturer's protocol. Briefly, cells (10^6) incubated in the presence of the examined compounds (DOX, PIRA and BP1) used at IC_{90} concentration were collected at indicated incubation times by centrifugation (300*g*, 5 min, 4°C). After washing

the cells with 1 ml staining buffer (Dulbecco's fetal bovine serum containing 0.2% BSA and 0.09% NaN_3), they were incubated in the dark for 20 min at 4°C with 100 µl staining buffer and 20 µl anti-Fas antibodies and then centrifuged and washed with 1 ml staining buffer. The cell pellet was resuspended in 500 µl staining buffer and analysed by flow cytometry (FACSCalibur, Becton Dickinson). Fas expression was determined by FL-1 measurements of 10^4 single events using a laser beam at 530/30 nm ($\lambda_{ex} = 488$ nm). Results were calculated using WinMDI (ver. 2.8) software.

Statistical analysis

Results are presented as the mean ± SD or median (interquartile range) of at least three independent experiments. Statistical analysis of the significance level of differences observed was carried out using either the parametric two-tailed Student *t*-test or the nonparametric Mann–Whitney *U*-test. *P* value of 0.05 or less was considered as a significant difference ($*P \leq 0.05$, $**P \leq 0.01$, $***P \leq 0.001$) between the compared groups.

Results

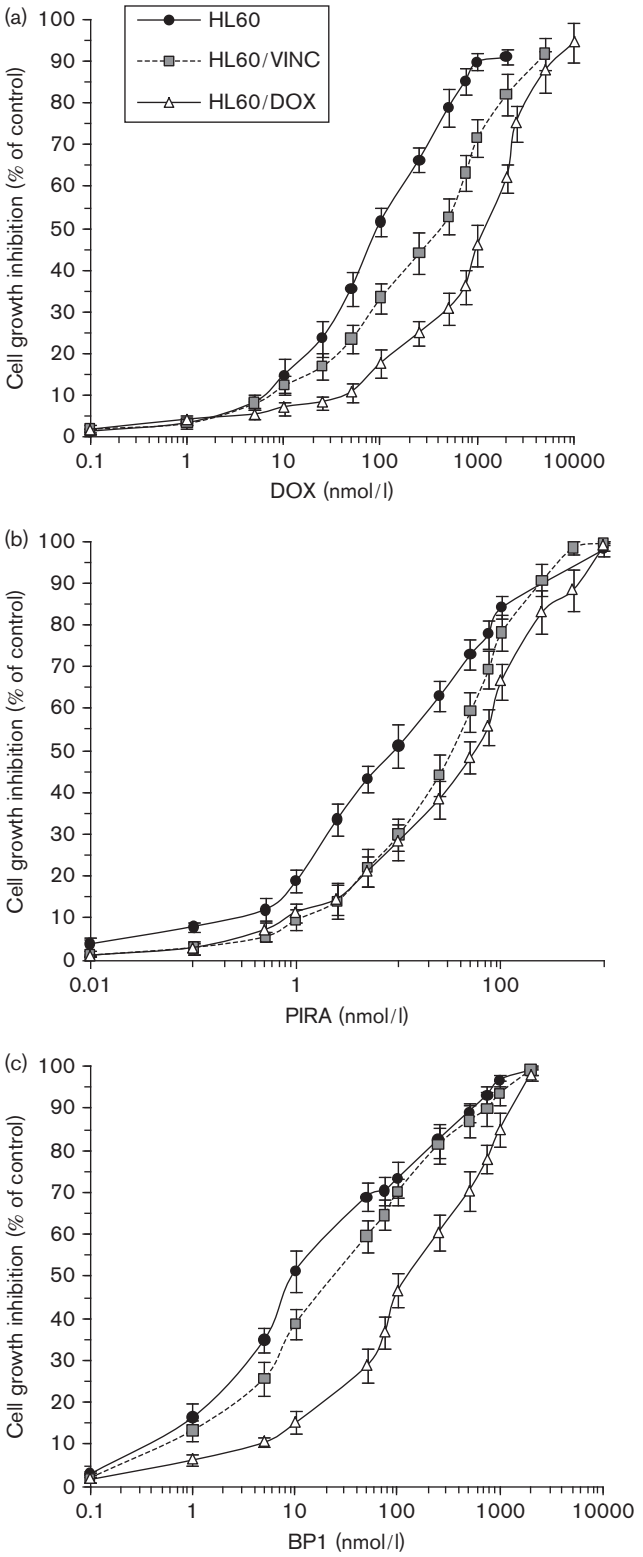
Ability of examined anthraquinone compounds to inhibit the growth of HL60, HL60/VINC and HL60/DOX cells

The ability of the examined compounds (DOX, PIRA and BP1) to inhibit the growth of the sensitive HL60 cell line and its MDR counterparts (HL60/VINC and HL60/DOX) was studied after the 72 h incubation (Fig. 2). The IC_{50} and IC_{90} values and resistance factor (RF) values determined for the examined compounds are shown in Table 1. HL60/DOX cells were seen to exhibit about two-fold to four-fold higher resistance to all studied agents compared with HL60/VINC cells. The most striking difference between the resistance of HL60/DOX and HL60/VINC was observed for BP1 (RF = 13.5 vs. RF = 3.5, respectively).

Intracellular accumulation of the examined anthraquinone compounds

Intracellular accumulation of the examined compounds (DOX, PIRA, BP1; 1 µmol/l) in parental HL60 and MDR (HL60/VINC and HL60/DOX) cells was measured by flow cytometry. Figure 3a shows the histograms obtained in typical experiments for examined samples after 15 min incubation of cells with compounds having high kinetics of cellular uptake (PIRA and BP1) [24,25] and 5 h incubation for DOX having low kinetics of cellular uptake [26]. For DOX and PIRA, an important decrease (by about 40%) in intracellular accumulation was observed in HL60/VINC and HL60/DOX cells in comparison with the parental HL60 line. In the case of BP1, more pronounced deficiency (by about 70%) in its intracellular accumulation in HL60/DOX was found. Interestingly, the intracellular retention of BP1 in HL60/VINC cells was similar to that observed for the sensitive HL60 line.

Fig. 2



Cytotoxic effect of the examined anthraquinone compounds on HL60, HL60/VINC and HL60/DOX cells. Cells were incubated for 72 h with 12 different concentrations of (a) doxorubicin (DOX), (b) pirarubicin (PIRA) and (c) benzoperimidine BP1. The data points represent mean ± SD.

Table 1 Cytotoxic activity of DOX, PIRA and BP1 towards the HL60 cell line and its multidrug-resistant sublines HL60/VINC and HL60/DOX

	IC_{50} (nmol/l)					IC_{90} (nmol/l)		
Compound	HL60	HL60/VINC	RF	HL60/DOX	RF	HL60	HL60/VINC	HL60/DOX
DOX	110 ± 18	509 ± 35	4.6	1606 ± 188	14.5	2001 ± 249	5001 ± 456	8001 ± 442
PIRA	8.1 ± 0.9	35.3 ± 5.0	4.4	60.3 ± 7.0	7.4	170 ± 22	250 ± 29	750 ± 43
BP1	10.0 ± 1.5	35.0 ± 5.1	3.5	130 ± 16	13.0	600 ± 64	750 ± 65	1200 ± 188

IC_{50} and IC_{90} values are the compound concentrations required to inhibit 50 and 90% of cell growth, respectively. Resistance factor (RF) was calculated as $RF = IC_{50}(R)/IC_{50}(S)$; S, sensitive HL60 cells, R, resistant HL60/VINC or HL60/DOX cells. The values represent mean ± SD of at least five independent experiments. BP1, benzoperimidine; DOX, doxorubicin; PIRA, pirarubicin.

Effect of examined anthraquinone compounds on cell cycle distribution

The effect of the studied compounds at IC_{50} (DOX, PIRA) and IC_{90} (DOX, PIRA, BP1) on HL60, HL60/VINC and HL60/DOX cell cycle distribution was examined for up to 96 h of incubation (Fig. 4).

It was found that cell cycle distribution and percentage of the apoptotic cell subpopulation of sensitive HL60 cells treated with DOX and PIRA used at IC_{50} concentration did not cause statistically significant changes in comparison with the control cell population (Fig. 4a and b). In their MDR counterparts, a decrease in the percentage of cells in G1 phase and fluctuations in S phase were observed, as well as G2/M arrest followed by an increase in the percentage of sub-G1 subpopulation, especially from 48 h of incubation. Interestingly, the most significant increase in the apoptotic subpopulation was observed for HL60/VINC cells incubated with DOX.

Incubation of cells with compounds at IC_{90} concentration resulted in significant alterations in cell cycle distribution and in a much more pronounced effect on the appearance of apoptotic cells. An important decrease in the percentage of cells in G1 phase and a very high G2/M blockage were observed (Fig. 4), except for BP1-exposed HL60/VINC cells showing a continuous decrease in the content of G2/M cells between 36 and 72 h of the experiment and some increase in this content found only at 96 h of incubation (Fig. 4c). Treatment of examined cells with compounds used at IC_{90} also resulted in a marked increase in the percentage of the subdiploid subpopulation starting from 36 h of incubation. However, similarly to the above-mentioned results found in the case of compounds used at IC_{50}, HL60/VINC cells incubated with the examined compounds at IC_{90} were more susceptible to apoptosis compared with HL60 and HL60/DOX cells. Interestingly, BP1 caused a marked increase in the number of subdiploid HL60/VINC cells from the beginning of the experiment (Fig. 4c).

Electrophoretic analysis of DNA degradation upon treatment with examined anthraquinone compounds

To determine the mode of DNA degradation of the examined cells upon treatment with DOX, PIRA and BP1, an agarose gel electrophoresis of DNA isolated from cells incubated for up to 96 h with the examined compounds used at IC_{90} concentration was conducted. In Fig. 5, 'DNA-ladders' obtained in a typical experiment are presented. As can be seen, in the case of all analysed cells (HL60, HL60/VINC and HL60/DOX), the examined compounds caused oligonucleosomal DNA fragmentation characteristic for apoptosis.

Caspase-3 activity induced by examined anthraquinone compounds

To determine the involvement of caspase-3 in apoptosis of sensitive HL60 and resistant HL60/VINC and HL60/DOX cells triggered by the examined compounds (DOX, PIRA, BP1), the cells were incubated for 72 h with these agents used at both IC_{50} and IC_{90} concentrations (Fig. 6a). In addition, cells incubated for 4 h after exposure to UVC irradiation (70 mJ/cm^2), a well-known inducer of caspase-3-dependent apoptosis [13], were taken as a positive control. Interestingly, caspase-3 activities were found to be the highest within HL60/VINC cells after treatment with DOX and PIRA (Fig. 6a). Surprisingly, the observed levels of caspase-3 activities were very similar and independent of the DOX and PIRA concentrations used (at IC_{50} or IC_{90}). Similar results were observed after treatment of HL60/VINC cells with BP1 (Fig. 6c). However, they showed about two-fold lower activity of this protein in comparison with the level found in response of these cells to DOX or PIRA treatment. The lowest activity of caspase-3 was observed in the case of HL60/DOX cells, independent of the agent used to induce apoptosis (DOX, PIRA, BP1 or UVC).

Caspase-8 activity induced by the examined anthraquinone compounds

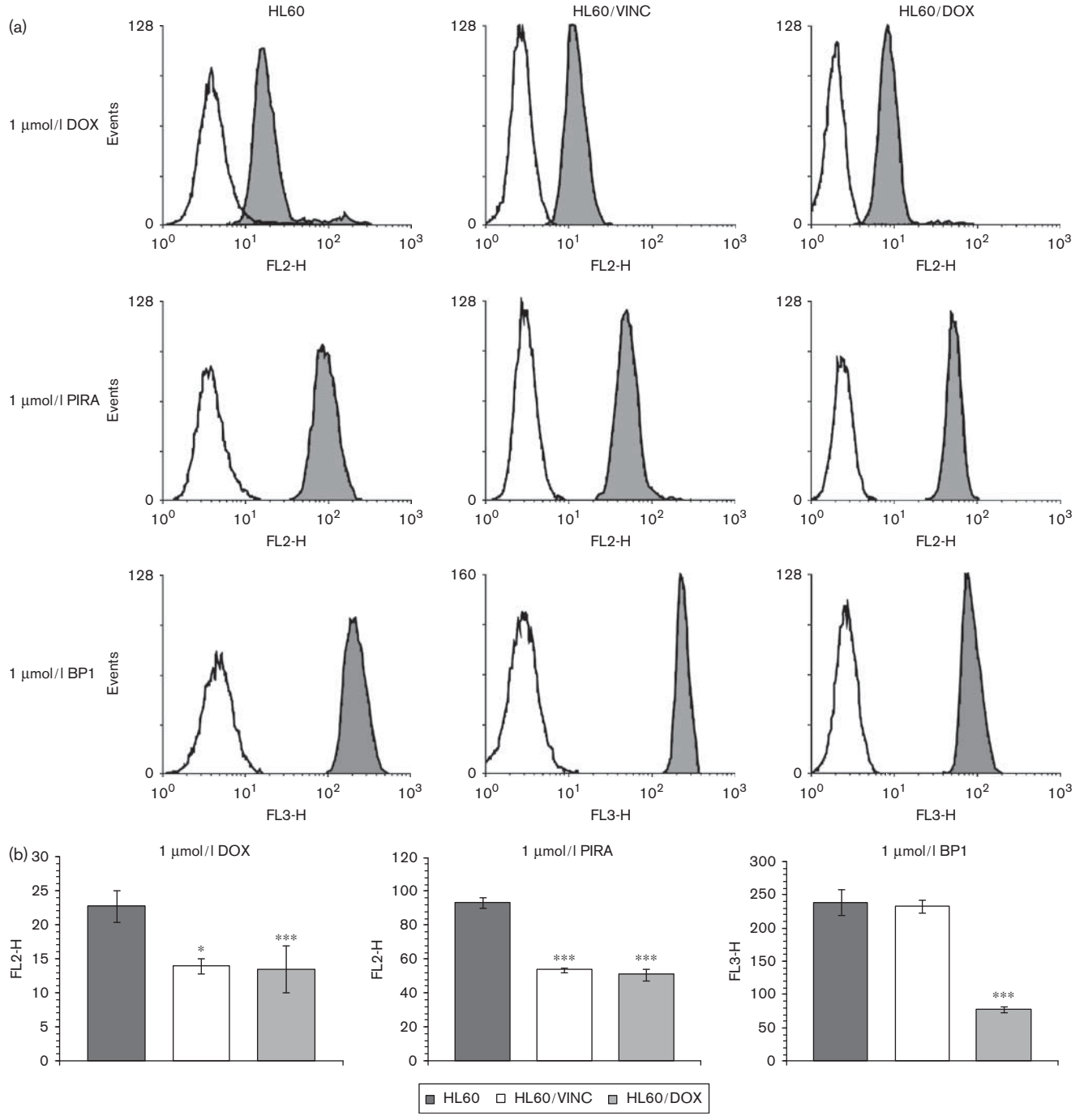
To evaluate the involvement of the extrinsic pathway in the activation of apoptosis of sensitive HL60 and resistant HL60/VINC and HL60/DOX cells in response to treatment with the examined compounds, the activity of caspase-8 in cells incubated up to 48 h with DOX, PIRA and BP1 used at IC_{90} concentration was determined (Fig. 6b). It was confirmed that this enzyme became active in a time-dependent manner in all studied cells. Interestingly, very similarly to the results obtained for caspase-3, the highest activity of caspase-8 was observed within HL60/VINC cells, especially after treatment with DOX and PIRA, whereas about two-fold to three-fold lower activity of this protein was observed after treatment

of HL60/VINC cells with BP1. In addition, it was seen that in the case of cell treatment with all studied compounds (DOX, PIRA and BP1), the activity of caspase-8 was very similar for HL60 and HL60/DOX cells.

ATP content in HL60, HL60/VINC and HL60/DOX cells

No statistically significant differences were observed between the ATP intracellular levels of cells in the logarithmic and steady-state phase of growth (Table 2).

Fig. 3



Intracellular accumulation of the examined anthraquinone compounds. Cells were incubated for 15 min with 1 μmol/l pirarubicin (PIRA) or benzoperimidine BP1 or for 5 h with 1 μmol/l doxorubicin (DOX) and analysed as described in the 'Materials and methods' section. (a) Histograms of a typical experiment (white, control cells; grey, cells incubated with compounds). (b) The data represent mean ± SD of at least four independent experiments. Asterisks indicate statistically significant differences (the Student *t*-test) between the accumulation values found for HL60/VINC or HL60/DOX and those found for parental HL60 cells.

Nevertheless, there are distinct differences in ATP level between the sensitive HL60 cell line and its MDR counterparts. Interestingly, it was found that HL60/VINC cells had about 1.3-fold decrease in ATP level, whereas HL60/DOX cells showed about 1.4-fold increase in comparison with values found for sensitive HL60 cells. However, it should be noted that the obtained results concern the total ATP content, not the intracellular concentration, and could result at least in part from differences in the volume of examined cells (HL60/VINC cells have smaller volume and HL60/DOX cells are larger than HL60 cells; data not presented).

Fig. 4

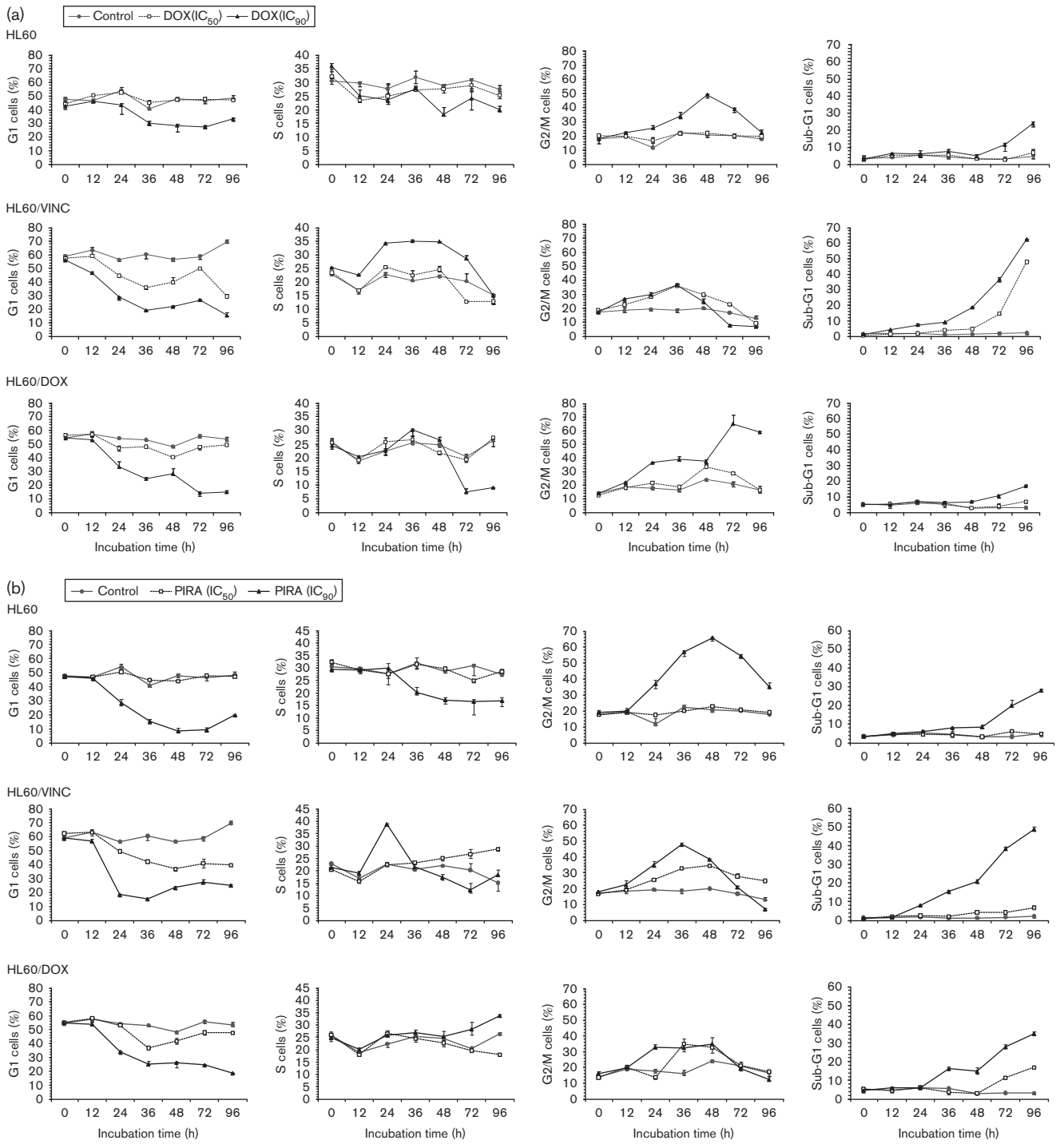


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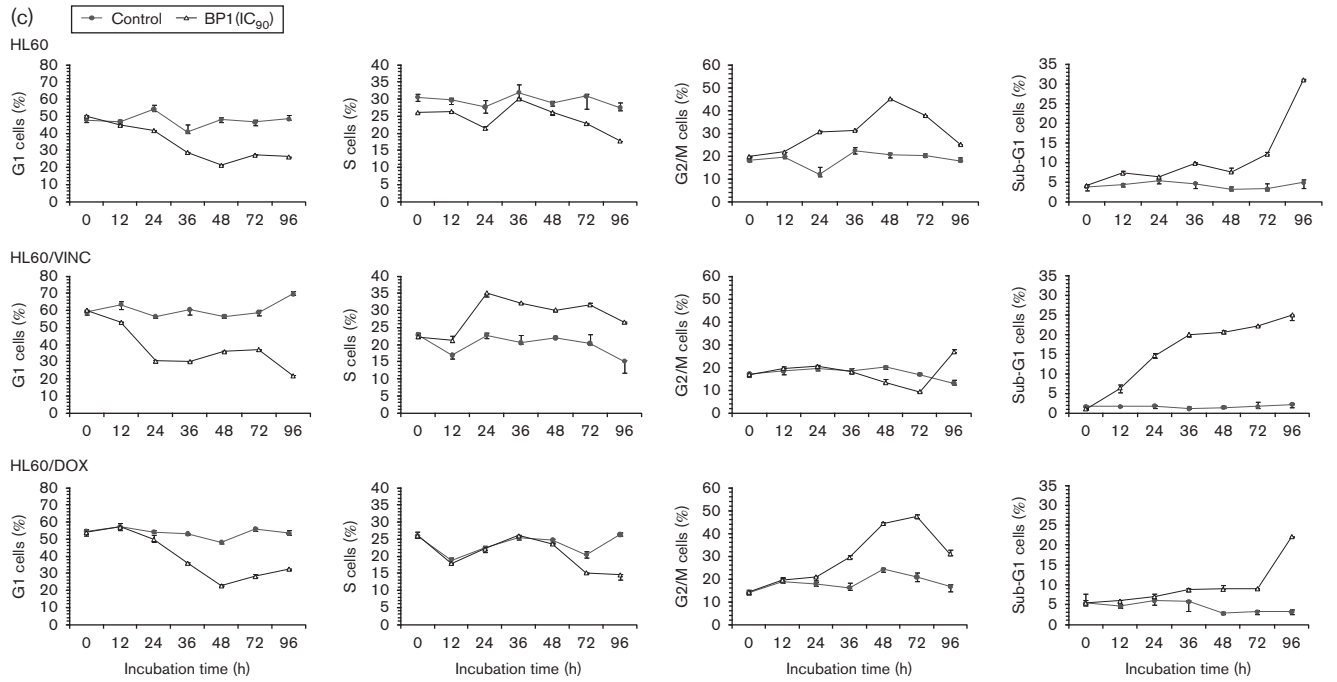
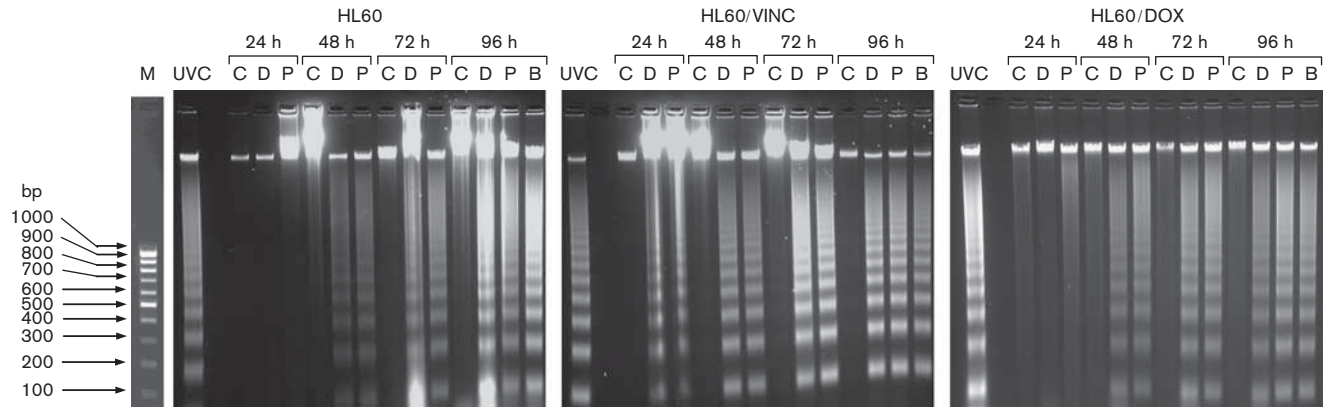
Fig. 4 *(Continued)*



Effect of the examined anthraquinone compounds on cell cycle distribution. Cells were incubated in the presence of (a) doxorubicin (DOX), (b) pirarubicin (PIRA) or (c) benzoperimidine BP1 at both IC_{50} and IC_{90} concentrations and analysed as described in the 'Materials and methods' section. Data presented as median (interquartile range) were determined from at least three independent experiments.

Fig. 5

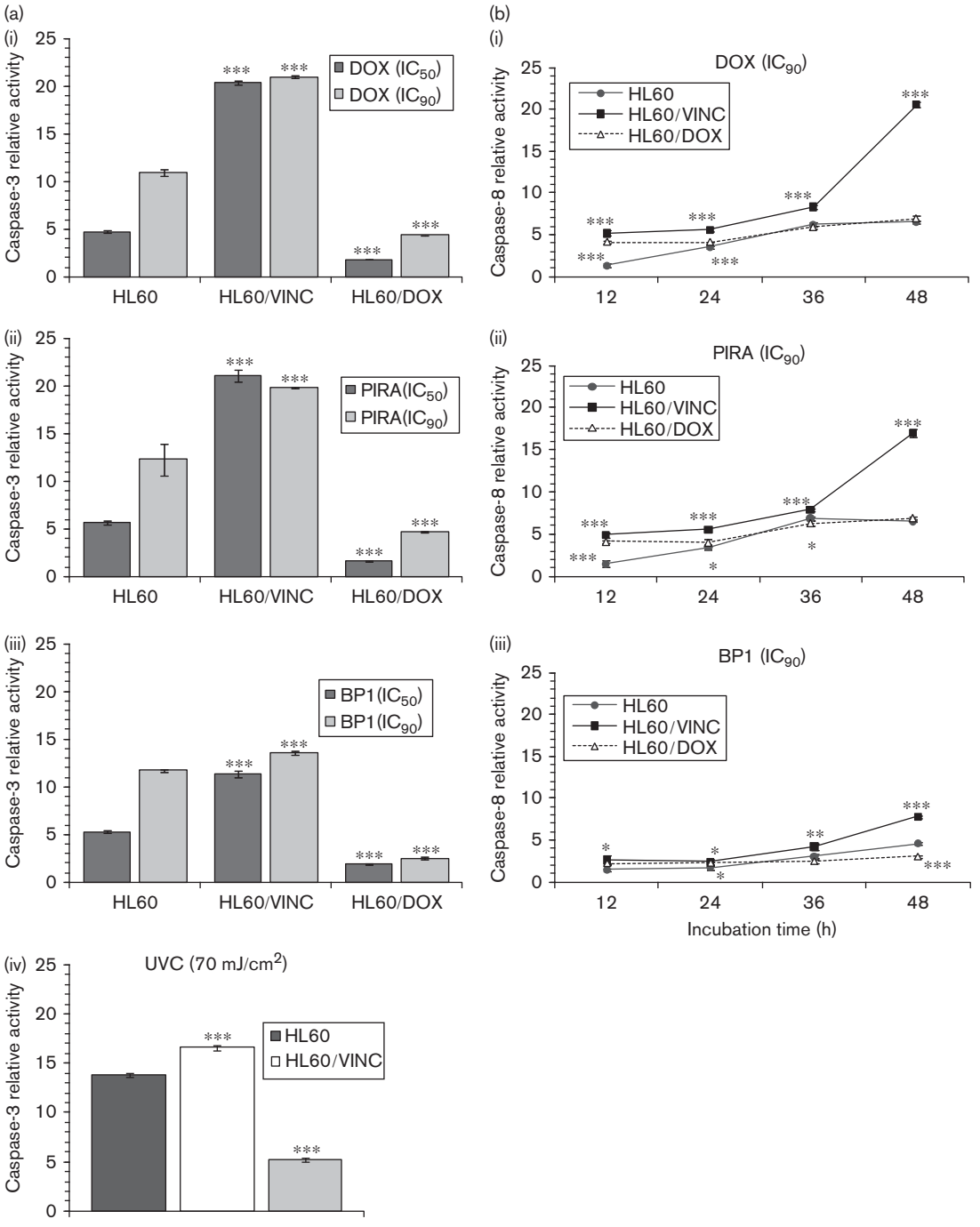


Electrophoretic analysis of DNA degradation upon treatment with the examined anthraquinone compounds. Typical electrophoretograms of the DNA isolated from control cells and cells incubated with the examined compounds. Cells (1.5×10^6) were incubated up to 96 h with doxorubicin (DOX), pirarubicin (PIRA) or benzoperimidine BP1 (IC_{90}). DNA was isolated and analysed as described in the 'Materials and methods' section. M, DNA size markers (100–1000 bp); UVC, DNA isolated from cells incubated for 4 h after UVC illumination (70 mJ/cm^2, positive control); C, DNA isolated from control cells; D, P and B, DNA isolated from cells incubated in the presence of DOX, PIRA and BP1, respectively.

Effect of the examined anthraquinone compounds on Fas receptor expression

Analysis of relative Fas (CD95) receptor expression on the surface of HL60, HL60/VINC and HL60/DOX cells incubated for 12, 24 and 48 h, respectively, with the examined compounds (DOX, PIRA or BP1) at IC_{90} showed no changes in Fas receptor expression caused by these agents (Fig. 7).

Fig. 6



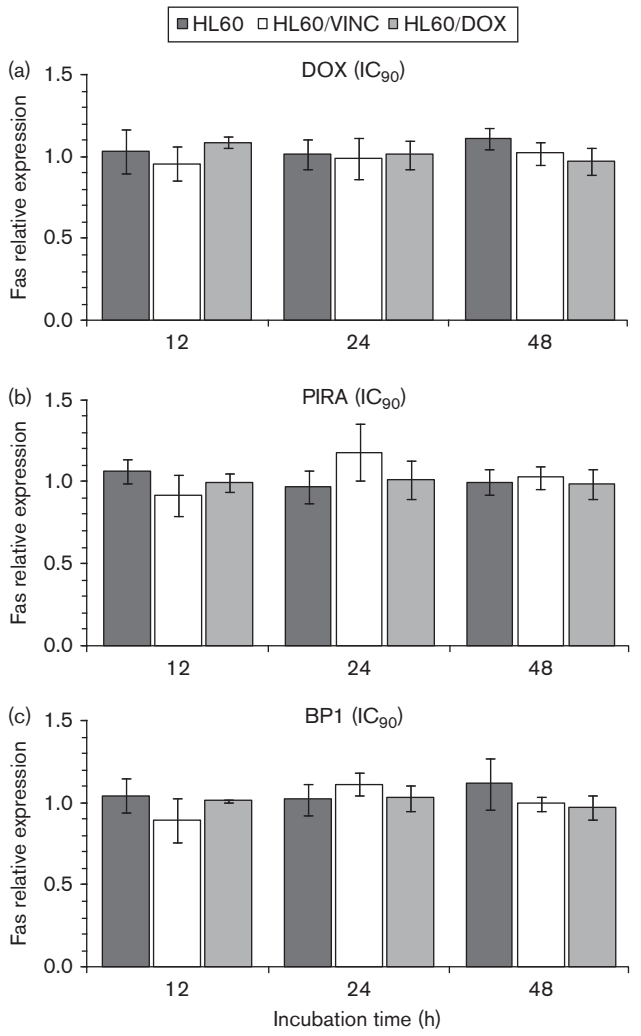
Caspase-3 and caspase-8 activities in cells after treatment with the examined anthraquinone compounds. (a) Caspase-3 activity in cells incubated with (i) doxorubicin (DOX), (ii) pirarubicin (PIRA), (iii) benzoperimidine BP1 and (iv) after UVC illumination (70 mJ/cm^2, positive control). (b) Caspase-8 activity in cells incubated with (i) DOX, (ii) PIRA or (iii) BP1. Cells (10^6) were treated as described in the 'Materials and methods' section. The caspase-3 and caspase-8 relative activities were found to be increased in $A_{405\,nm}$ of the lysate obtained from cells incubated in the presence of the examined compound by a given factor in relation to the value found for the lysate obtained from cells incubated in the absence of the compound (control cells). The data represent mean ± SD of at least three independent experiments. The asterisks (the Student *t*-test) indicate values that are significantly different from values obtained for parental HL60 cells.

Table 2 ATP content in sensitive HL60 as well as multidrug-resistant HL60/VINC and HL60/DOX cells

	ATP (nmol/10^6 cells)		
Phase of cell growth	HL60	HL60/VINC	HL60/DOX
Logarithmic	6.5 ± 0.9	4.9 ± 0.7	9.0 ± 1.0
Steady-state	6.4 ± 1.3	5.1 ± 0.8	9.1 ± 0.6

The values represent mean ± SD of at least five independent experiments. DOX, doxorubicin.

Fig. 7



Cell surface expression of Fas receptor. Cells were incubated with (a) doxorubicin (DOX), (b) pirarubicin (PIRA) or (c) benzoperimidine BP1 at IC_{90} concentration and analysed as described in the 'Materials and methods' section. Fas expression was shown as the relative fluorescence of cells stained with anti-Fas monoclonal antibodies after treatment with the examined agents in relation to the fluorescence of cells incubated in the absence of the compound (control cells). The data represent mean ± SD of at least three independent experiments.

Discussion

The role of ATP-binding cassette transporters in inhibiting apoptosis of multidrug-resistant tumour cells is not clear and seems to depend strongly on properties that are specific to tumour type and on apoptotic stimuli.

In this study, we examined the effect of three selected anthraquinone antitumour agents – DOX, PIRA and benzoperimidine BP1 – on inducing apoptosis of the human promyelocytic sensitive leukaemia HL60 cell line and its MDR sublines exhibiting two different phenotypes of MDR related to the overexpression of P-gp (HL60/VINC) or MRP1 (HL60/DOX). DOX and its derivative PIRA (having the tetrahydropyranyl substituent at 4′-OH position) are among the most effective drugs currently available for the treatment of various human neoplastic diseases including leukaemias, lymphomas and solid tumours [27,28]. However, the clinical usefulness of these drugs (especially DOX) is limited by the occurrence of MDR [29,30]. Benzoperimidines (among them BP1) belong to a novel group of synthetic anthraquinone agents of potential clinical interest because of their ability to overcome MDR of some tumour cells [18,25,31].

It was found that HL60/DOX cells exhibited about two-fold to four-fold higher resistance to all the studied agents (DOX, PIRA and BP1) compared with HL60/VINC cells. However, the differences observed in their cellular accumulation level may only in part explain the decrease in the cytotoxic activity of these compounds against multidrug-resistant HL60/VINC and HL60/DOX sublines in comparison with the parental HL60 cell line. In fact, on the basis of the results found for ATPase mutant P-gp cells, it was proposed that P-gp can protect against cell death by a dual function, which involves not only ATPase-dependent drug efflux leading to the decrease in its intracellular accumulation but also ATPase-independent inhibition of apoptosis [32].

All examined anthraquinone agents used at IC_{50} and IC_{90} concentrations were able to influence the cell cycle of sensitive HL60 and resistant (HL60/VINC and HL60/DOX) cells and induce apoptosis. It is well known that these agents induce serious cellular DNA damage caused by DNA intercalation, free radical formation, alkylation of DNA and DNA crosslinking [28]. In response to DNA damage, cellular pathways are activated in order to activate repair mechanisms. It leads to G1/S or G2/M cell cycle arrest depending on their p53 status [33,34]. Many cancer cells, including promyelocytic leukaemia HL60 cells, are known to lack a functional p53 protein [35–37] causing the abrogation of the effective G1/S checkpoint [33]. Therefore, as presented in the study, the incubation of HL60 cells and their MDR counterparts with the examined anthraquinone compounds (DOX, PIRA and BP1) resulted only in G2/M blockage followed by an increase in percentage of the apoptotic cell subpopulation.

Interestingly, the studied cells exhibited a differential sensitivity to undergo apoptosis. It was seen that HL60/VINC

cells were more susceptible to undergo apoptosis induced by the studied agents compared with HL60 and HL60/DOX cells. Moreover, despite the role of MDR pumps in inhibiting caspase-dependent apoptotic pathways in many resistant tumour cells, our results have shown that all agents examined (independently of their structure and retaining activity against MDR-resistant cells) triggered both caspase-3 and caspase-8 activation of apoptosis of the sensitive HL60 cell line and MDR sublines. Our results are consistent with data found by Choi *et al.* [38] in the study examining the apoptosis of HL60 cells and two MDR variant sublines (HL60/VINC and HL60/DOX) mediated by proteasome inhibitor Z-Ile-Glu(OtBu)-Ala-Leu-aldehyde (PSI). They demonstrated that processing of caspase-3 and caspase-8 activation in multidrug-resistant HL60/VINC and HL60/DOX cells occurred in a manner comparable to that of sensitive HL60 cells, indicating that the apoptosis machinery can still be engaged in the drug-resistant cells, although higher concentrations of PSI were required to achieve this effect. Our results are also in line with data reported by Bielak-Żmijewska *et al.* [13] showing that the overexpression of P-gp and MRP1 did not inhibit caspase-3-dependent apoptosis of HL60/VINC and HL60/DOX cells induced by curcumin and UVC irradiation. Furthermore, it is of interest to note that the highest activities of both caspase-3 and caspase-8 were observed in our study within HL60/VINC cells treated with the examined anthraquinone compounds (especially DOX and PIRA). These results are consistent with the data obtained by Mantovani *et al.* [17] suggesting that caspase-3 cleavage of P-gp would be a proapoptotic self-enhancing mechanism favouring the apoptosis of tumour cells overexpressing P-gp. Moreover, it was reported very recently that P-gp can stimulate caspase-3-dependent apoptosis of tumour cells by the induction of miR-16 targeting the 3′-untranslated region of Bcl-2 mRNA and thus suppressing the expression of Bcl-2 protein (a key antiapoptotic agent that prevents caspase-3 activation) by blocking mitochondrial cytochrome c release [39]. It fact, it was demonstrated in this elegant study that the knockdown of P-gp by *mdr1*-specific antisense oligonucleotides led to the upregulation of *Bcl-2* expression, inhibition of procaspase-3 cleavage and significant reduction of radiation-induced apoptosis in hepatocellular carcinoma RHepG2 cells. In contrast, the transient transfection of RHepG2 cells with *mdr1* expression vector induced the expression of miR-16, decreased the Bcl-2 level and enhanced caspase-dependent apoptosis. In addition, experimental data obtained by the authors with the aid of verapamil (a well-known modulator of P-gp blocking the efflux of P-gp substrates by this MDR transporter) showing that this agent decreased radiation-induced apoptosis significantly, accompanied by the downregulation of miR-16 and increased expression of Bcl-2, proved that the involvement of P-gp in apoptosis induction is related to its efflux pump activity. Thus, the regulation of miR-16/Bcl-2 expression by P-gp is believed to be associated with the efflux of P-gp substrates involved in intracellular signalling pathways and gene expression that are yet to be identified. Similar results were also found for MRP1 indicating that this MDR transporter could also be involved in regulating Bcl-2 expression in resistant tumour cells [40]. It was reported that small lung H69AR resistant cancer cells overexpressing MRP1 were much more susceptible to undergo a caspase-dependent apoptosis than parental H69 cells and that this high propensity was related to the downregulation of Bcl-2 (H69AR was characterized by an almost undetectable Bcl-2 level).

ATP is a cellular factor required for both MDR pump functionality and proper execution of apoptosis [3,4,41,42]. However, no correlation was found between ATP intracellular level in the examined sensitive and MDR cells and their ability to undergo apoptosis triggered by the examined anthraquinone agents (HL60/VINC cells exhibiting strikingly high sensitivity to undergo apoptosis contained a relatively low intracellular level of ATP in comparison with its content found in HL60 and HL60/DOX cells that are less sensitive to apoptosis). It suggests that the inhibition of apoptosis observed in some tumour cells (among them sensitive HL60 and resistant HL60/VINC and HL60/DOX cells) is not related to the decrease in ATP level.

Apoptosis induction mediated through the Fas (CD95) death receptor is one of the best-defined apoptotic pathways occurring frequently in tumour cells in response to chemotherapy [43,44]. However, the data obtained in the present study show that, although the induction of apoptosis triggered by the examined anthraquinone agents occurred with the involvement of the caspase-8-dependent extrinsic pathway, they did not change the expression of Fas on the surface of HL60 sensitive and resistant (HL60/VINC and HL60/DOX) cells. Nevertheless, some changes in Fas activity could not be excluded without changing the quantity of this receptor. Our results are consistent with data obtained by Notarbartolo *et al.* [45] reporting that the Fas/FasL pathway did not mediate the apoptotic effects of DOX in HL60 and HL60R cells overexpressing P-gp. Similar data were also reported for a large series of P-gp-expressing and MRP-expressing drug-resistant leukaemia sublines, pointing out that MDR pumps did not confer resistance to Fas-induced apoptosis [14].

In summary, taking into account the complexity of molecular events leading to cell death, there is no doubt that further research is needed for better understanding the reported data aimed at elucidating the role of MDR pumps in inhibiting apoptosis of leukaemia cells. It should be focused more precisely on examining specific mechanisms involved in extrinsic and intrinsic apoptotic pathways triggered by anthraquinone antitumour agents in the studied

HL60-sensitive line and its resistant cells overexpressing MDR pumps, as well as in other leukaemia cells. Moreover, for the development of more effective therapeutic strategies, other mechanisms involved in the MDR phenomenon (e.g. intensification of DNA repair pathways, alterations of drug targets, increased biotransformation and/or inactivation of drugs) [46,47] should be considered for the elucidation of complex cellular mechanisms leading to changes in response of MDR cells to various apoptotic stimuli.

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Conflicts of interest

There are no conflicts of interest.

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