Role of structural factors of antitumour anthraquinone derivatives and analogues in the ability to undergo bioreductive activation by NADPH cytochrome P450 reductase. Implications for increasing the activity against sensitive and multidrug-resistant leukaemia HL60 cells

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The aim of this study was to examine the role of structural factors of antitumour anthraquinone derivatives and analogues in the ability to undergo bioreductive activation by NADPH cytochrome P450 reductase (CPR) and determine the impact of this activation on increasing the activity especially with regard to multidrug resistant (MDR) tumour cells. It was found that at a high NADPH concentration (500 μmol/l), the anthracenedione agent ametantrone, with an unmodified quinone structure, was susceptible to CPR-dependent reductive activation. In contrast, it was shown that compounds with modified quinone grouping (benzoperimidine BP1, anthrapyridone CO1 and pyrazolopyrimidoacridine PPAC2) did not undergo reductive activation by CPR. This suggests that the presence of a modified quinone function is the structural factor excluding reductive activation of antitumour anthraquinone derivatives and analogues by CPR. In the second part of the work, the ability of antitumour anthraquinone derivatives and analogues to inhibit the growth of the human promyelocytic, sensitive leukaemia HL60 cell line as well as its MDR sublines exhibiting two different phenotypes of MDR related to the overexpression of P-glycoprotein (HL60/VINC) or MRP1 (HL60/DOX) was studied in the presence of exogenously added CPR. A significant increase in the activity of ametantrone with an unmodified quinone structure after its reductive conversion by CPR was observed against HL60 as well as HL60/VINC and HL60/DOX cells, whereas in the case of quinone-modified compounds (BP1, CO1 and PPAC2), the presence of the activation system had no effect on their activity against the sensitive and MDR tumour cells examined. *Anti-Cancer Drugs* 23:393–405 © 2012 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

The clinical usefulness of bioreductive antitumour drugs is limited by the occurrence of multidrug resistance associated with the presence of membrane transporters (e.g. P-glycoprotein, MRP1) belonging to the ATP-binding cassette protein family [1–3]. 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 [3,4].

There is an increasing body of evidence indicating that the reductive activation of antitumour drugs, for example, anthracyclines, mitomycin C, tirapazamine and indoloquinones could result in the formation of reactive intermediates capable of alkylation or crosslinking binding with DNA [5–10] and may lead to a significant increase in the cytotoxic activity of these compounds against tumour cells [7,10–13]. Furthermore, our previous results found for the anthracycline drug doxorubicin (DOX) and the anthracenedione agent mitoxantrone (MX) [14,15] suggest that the bioreductive activation of antitumour compounds belonging to the anthraquinone family could generate reactive intermediates able to irreversibly bind to DNA before being removed from resistant cells by MDR exporting pumps and provide a possibility to restore the activity of these drugs against multidrug-resistant tumour cells. In addition, it has been shown that the amount of NADPH is an essential factor determining the route of DOX as well MX activation by NADPH cytochrome P450 reductase (CPR) [14,15]. Similar findings were also reported for tirapazamine activation by this enzyme [11,12,16]. Thus, it is proposed that NADPH could participate in forming a coupled interactive system and, as a consequence, constitutes a control point in drug activation by cellular

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oxidoreductases. Our results showed that at a low NADPH concentration, DOX and MX underwent only the redox cycling by CPR related to the formation of an unstable semiquionone radical, followed by the production of a superoxide anion radical (O_2•). In contrast, at a high NADPH concentration, the drugs underwent multistage chemical transformations of the chromophore part in the case of DOX or side chains containing reactive secondary aminoalkyl groups in the case of MX. In addition, it was found, using superoxide dismutase (SOD), that the first stage, according to the mechanism of the redox cycling of examined drugs (DOX and MX), was of major importance for their metabolic conversion [14,15].

Moreover, in our recent study aimed at elucidating the role of structural factors of antitumour anthracycline drugs in the ability to undergo bioreductive activation by CPR, it was found that anthracyclines with an unmodified quinone structure, daunorubicin (DR), idarubicin (IDA) and pirarubicin (PIRA), were susceptible upon CPR catalysis to multistage chemical transformation of the chromophore part. In contrast, it was found that 5-iminodaunorubicin (5-Im-DR), with a modified quinone grouping as the only structural change with regard to the parental drug (DR), lost this ability. This suggests that the presence of a modified quinone function is the structural factor excluding the reductive activation of anthraquinone compounds (at least anthracycline agents) by CPR [17].

To verify and generalize our supposition, in the present study, we examined the ability of several antitumour anthraquinone derivatives and analogues to undergo bioreductive activation by CPR and determined the impact of this activation on increasing the activity against the human promyelocytic sensitive leukaemia HL60 cell line as well as its MDR sublines exhibiting two different phenotypes of MDR related to the overexpression of P-glycoprotein (HL60/VINC) or MRP1 (HL60/DOX). The selected compounds belong to various chemical groups of bioreductive antitumour agents: anthracenediones [ametantrone (AMET)], benzoperimidines (BP1), anthrapyridones (CO1) and pyrazolopyrimidoacridines (PPAC2).

Materials and methods

Reagents

IDA, NADPH, SOD and vincristine were obtained from Sigma-Aldrich (Saint Louis, Missouri, USA). DOX was a gift from Pharmacia-Upjohn (Milan, Italy). AMET [18], benzoperimidine BP1 [19], anthrapyridone CO1 [20] and pyrazolopyrimidoacridine PPAC2 [21] were synthesized according to the procedures published elsewhere. CPR from human liver was obtained in C. Roland Wolf's laboratory, Ninewells Hospital and Medical School (Dundee, UK) according to the procedure described earlier [22].

Cell culture

The human promyelocytic leukaemia HL60 line (Division of Biology, Kansas State University, Manhattan, Kansas, USA) and its resistant sublines HL60/VINC (overexpressing P-glycoprotein) [23] and HL60/DOX (overexpressing MRP1) [24,25] 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% FBS (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, HL60/DOX) initiated at a density of 10^5 cells/ml or 5×10^5 cells/ml grew 72 or 24 h for steady-state or logarithmic phase of growth, respectively. They were counted before the assay using a Burker haemocytometer (Marienfeld-Superior, Lauda-Königshofen, Germany). Cell viability was assessed by trypan blue exclusion.

Enzymatic studies

Stock solutions of examined compounds and NADPH ($C_0 = 10^{-3}$ mol/l) were prepared just before use. Concentrations were determined by diluting stock solutions in water to approximately 50 μmol/l using characteristic values of maximum absorption wavelengths (λ_{max}) and extinction coefficients (ε) for these compounds given in Table 1.

The reaction mixtures in 0.01 mol/l K_2HPO_4/KH_2PO_4 buffer (pH 7.25) contained 100 μmol/l examined compound, 100 or 500 μmol/l NADPH, 4 μg/ml CPR and 0 or 500 U/ml SOD, respectively. All the reactions were initiated by the addition of CPR and conducted at 37°C. Absorption spectra of tested compounds were recorded at the indicated time points in the visible region (330–800 nm). NADPH oxidation was measured at $\lambda = 340$ nm using an extinction coefficient of $\varepsilon = 6220$/mol/l × cm. Absorption measurements were made on a Marcel E330 spectrophotometer (Marcel, Warsaw, Poland).

Cytotoxicity assays

For each cell line, the cytotoxic effects of examined compounds were determined by incubating cells (10^4) with 10 different concentrations of the compound for 72 h in standard 96-well plates. The enzymatic samples contained for the tested compound acting in redox cycling: 100 μmol/l examined compound, 100 μmol/l NADPH and 4 μg/ml CPR; for the compound incubated in the presence of activating system: 100 μmol/l examined compound, 500 μmol/l NADPH, 4 μg/ml CPR and 0 or 500 U/ml SOD (0.01 mol/l K_2HPO_4/KH_2PO_4 buffer, pH 7.25 at 37°C). The appropriate volumes of the enzymatic sample were added directly to the cell suspensions to yield concentrations varying in the following ranges: 0.01–500 nmol/l (IDA), 0.1 nmol/l–10 μmol/l (AMET), 0.1 nmol/l–1 μmol/l (BP1), 0.1 nmol/l–3 μmol/l (CO1) and 0.01 nmol/l–1 μmol/l (PPAC2), respectively, for all tested cells. Control assays were carried out for buffer alone at the highest percentage used in culture medium (2%, v/v)

Table 1 Spectroscopic characteristic of the studied compounds

Compound	λ_{max} (nm)	ε [1/(mol/l × cm)]	λ_{ex} (nm)	λ_{em} (nm)
IDA	480	11 500	480	590
AMET	638	7440	586	660
BP1	516	9800	535	665
CO1	495	6013	490	566
PPAC2	340	11 300	340	420
NADPH	340	6220	340	460

ε, extinction coefficient; λ_{em}, emission wavelength; λ_{ex}, excitation wavelength; λ_{max}, maximum absorption wavelength; AMET, ametantrone; BP1, benzoperimidine; CO1, anthrapyridone; IDA, idarubicin; PPAC2, pyrazolopyrimidoacridine.

and for NADPH alone (50 µmol/l) or CPR alone (0.4 µg/ml) at the highest concentrations used in in-vitro studies. The cell growth was determined by counting the viable cells in the presence of trypan blue using a Burker haemocytometer.

Cellular drug uptake

The cellular uptake of selected compounds (IDA, BP1 and CO1) alone (nonactivated), acting in the redox cycling and incubating in the presence of the activating system (without SOD or in the presence of SOD, respectively), was followed by monitoring the changes in the fluorescence signal. The characteristic values of extinction and emission wavelengths (λ_{ex} and λ_{em}) for these compounds are given in Table 1. The incubation of cells with the compound proceeded without compromising cell viability. All experiments were conducted in 1 cm quartz cuvettes containing 2 ml of 20 mmol/l HEPES buffer plus 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. In a typical experiment, 2×10^6 cells in the logarithmic growth phase were suspended in 2 ml of HEPES buffer under vigorous stirring. At t_0, 20 µl of the stock compound solution (for 'compound-alone' assays) or 20 µl of an appropriate enzymatic sample was quickly added to this suspension, yielding a concentration equal to 1 µmol/l. The decrease in the fluorescence intensity, F, at the appropriate emission wavelength characteristic for each compound (Table 1) was followed as a function of time until the curve $F = f(t)$ reached a plateau. All fluorescence measurements were made on a Perkin-Elmer LS 50B spectrofluorimeter (Waltham, Massachusetts, USA).

Intracellular accumulation of examined compounds determined by flow cytometry

Cells in the 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 compound solution (for 'compound-alone' assays) or 10 µl of an appropriate enzymatic sample was added to this suspension, yielding a concentration equal to 1 µmol/l, and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 15 min in the case of IDA, BP1 and CO1 or 5 h in the case of AMET, respectively. 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 conducted within the FL-2 fluorescence channel (bandpass filter, $\lambda = 585 \pm 21$ nm) for IDA and CO1 or within the FL-3 fluorescence channel (longpass filter, $\lambda \geq 670$ nm) in the case of BP1 after an excitation with the argon-ion laser ($\lambda_{ex} = 488$ nm) or within the FL-4 fluorescence channel (bandpass filter, $\lambda = 661 \pm 8$ nm) after an excitation with the red-diode laser ($\lambda_{ex} = 635$ nm) for AMET, respectively. For each experimental point, the fluorescence signal of 1×10^4 cells was measured. The data were analysed using BD CellQuest Pro (Becton Dickinson) as well as WinMDI (ver. 2.8) software *http://www.methods.info/software/flow/winmdi.html*.

Interaction of examined compounds with naked DNA

High-molecular-weight calf thymus DNA was dissolved in PBS buffer for 3 h under vigorous stirring. DNA concentration was determined from absorbance measurements at 260 nm using an extinction coefficient $\varepsilon_{260} = 13\,200$/mol/l × cm (bp). The interaction of selected compounds: IDA, BP1 and CO1 (1 µmol/l) alone (nonactivated), acting in the redox cycling and incubated in the presence of the activating system (in the absence or in the presence of SOD) with naked DNA, were examined by fluorimetric titration using a spectrofluorimetric method [26]. All experiments were conducted in 1 cm quartz cuvettes containing 2 ml of 20 mmol/l HEPES buffer plus 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. At t_0, 20 µl of the stock compound solution (for 'compound-alone' assays) or 20 µl of an appropriate enzymatic sample was quickly added to 2 ml of HEPES buffer, yielding a concentration equal to 1 µmol/l. The binding of examined compounds to DNA was followed by monitoring the decrease in the fluorescence signal at the fluorescence intensity F at the appropriate emission wavelength characteristic for each compound (Table 1) after the addition of 2 µmol/l DNA portions. The fluorescence measurements were made on a Perkin-Elmer LS 50B spectrofluorimeter.

Statistical analysis

Results are presented as the mean ± SD of at least five independent experiments. Statistical analysis of the significance level of the differences observed between analysed values found for nonactivated and activated compounds was carried out using Student's t-test. P-value less than 0.05 was considered as a significant difference.

Results

The structures of examined compounds belonging to antitumour anthraquinone derivatives and analogues: anthracenediones (AMET) benzoperimidines (BP1), anthrapyridones (CO1) and pyrazolopyrimidoacridines (PPAC2)

are presented in Fig. 1. In addition, the anthracycline agent IDA was taken in the study as a reference positive compound exhibiting a well proven ability to undergo CPR-dependent reductive activation [17].

Reduction of examined compounds by NADPH cytochrome P450 reductase: spectroscopic studies

Figure 2 illustrates the representative absorption spectra of samples recorded during the incubation of 100 μmol/l examined compound (IDA, AMET, BP1, CO1 or PPAC2, respectively) with 4 μg/ml CPR in the presence of NADPH at a low concentration (100 μmol/l) or at a high concentration (500 μmol/l). Figure 2a also presents the results found for the samples containing 100 μmol/l IDA (reference compound), 500 μmol/l NADPH, 4 μg/ml CPR and additionally 500 U/ml SOD. The absorption measurements at 340 nm characteristic for NADPH and maximum absorption wavelengths characteristic for studied compounds (Table 1) were also carried out continuously for each sample studied.

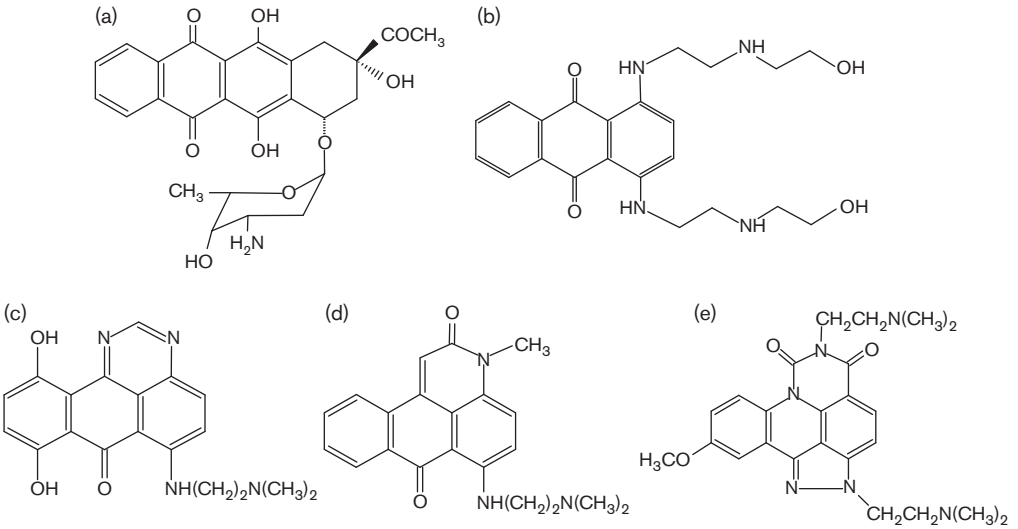
For each examined compound, it was found that at a low NADPH concentration (100 μmol/l), a significant decrease in the absorption intensity at 340 nm ($A_{340\,nm}$) was observed immediately after the addition of CPR. This indicates that all studied agents caused a high stimulation of NADPH oxidation catalysed by CPR. However, no changes were observed in the absorption spectra of the studied compounds up to 3 h (the presented data show the absorption spectra recorded for the first 60 min only). In contrast, at a high NADPH concentration (500 μmol/l) in the case of the reference compound IDA with an unmodified quinone structure, after the addition of CPR, besides the decrease in $A_{340\,nm}$, a significant decrease in $A_{480\,nm}$ was also observed, followed by further modifications of absorption spectra of this compound, indicating the modifications in the chromophore part of its structure. Nevertheless, the addition of SOD to the enzymatic samples containing CPR and high concentrations of NADPH (500 μmol/l) abolished the chemical transformations of IDA (only the rapid oxidation of NADPH occurred without changes in the absorption spectra of IDA; Fig. 2a).

In the case of AMET with an unmodified quinone structure and other compounds possessing quinone-modified grouping (BP1, CO1 and PPAC2) at a high NADPH concentration (500 μmol/l), immediately after the addition of CPR, a significant decrease in the absorption intensity at 340 nm ($A_{340\,nm}$) was observed but in contrast to the results obtained for anthracycline reference compound IDA, no changes in their absorption spectra were observed in the presence of the activating system (Fig. 2b–e), indicating the lack of modifications in the chromophore part of these compounds.

The activity of examined compounds towards the HL60 cell line and its multidrug-resistant sublines: HL60/VINC and HL60/DOX

The ability of the examined compounds (IDA, AMET, BP1, CO1 or PPAC2, respectively) to inhibit the growth of the human promyelocytic leukaemia HL60 cell line as well as its MDR sublines exhibiting two different phenotypes of MDR related to the overexpression of P-glycoprotein (HL60/VINC) or MRP1 (HL60/DOX) was studied in the presence or in the absence of exogenously

Fig. 1



Structures of examined compounds. (a) idarubicin (IDA), (b) ametantrone (AMET) (c) benzoperimidine (BP1), (d) anthrapyridone (CO1), (e) pyrazolopyrimidoacridine (PPAC2).

added NADPH and CPR. All control cells proliferated during 72 h. The growth rate of the parent cells (HL60) was comparable with both resistant cells used in the study (HL60/VINC and HL60/DOX). All cultures initiated at a density of 10^5 cells/ml grew to about 10^6 cells/ml (control count) in 72 h. The cytotoxic effect of examined compounds was determined by incubating cells (10^5) with 10 different concentrations of these compounds for 72 h. In calculating cell growth (percentage of control), untreated cells were used as the control. The results obtained for each cell line are illustrated in Fig. 3. Additional assays carried out for buffer alone, NADPH alone or CPR alone at the highest concentrations used in in-vitro studies showed that these agents had no effect on cell growth. One hundred per cent of control HL60, HL60/VINC and HL60/DOX cell growth was observed at the highest percentage of buffer used in the culture medium (2%, v/v) as well as at the highest concentrations of NADPH (50 μmol/l) or CPR (0.4 μg/ml) used. Control assays carried out for enzymatic samples containing both NADPH and CPR at the same highest concentrations showed that they neither had any effect on cell growth (100% of control cell growth was also observed, data not presented).

Our results (Fig. 3) showed that the incubation of HL60 sensitive and resistant (HL60/VINC and HL60/DOX) cells with all examined compounds (IDA, AMET, BP1, CO1 or PPAC2, respectively) pretreated in the presence of CPR and a low NADPH concentration (100 μmol/l) had no effect in increasing its activity in comparison with the drug alone. In contrast, the incubation of HL60 cells with agents with an unmodified quinone structure (IDA and AMET) pretreated with CPR at a high NADPH concentration (500 μmol/l) resulted in a significant increase in cell growth inhibition. The increasing effect on the cytotoxic activity of these compounds remained even when the drug (IDA or AMET, respectively) was added after a 10-min preincubation step with CPR and NADPH (data not presented). This effect was completely abolished in the presence of 500 U/ml SOD added to the enzymatic sample (Fig. 3a–b). Similar results were reported in our previous study for other antitumour agents with a nonmodified structure (anthracyclines: DOX, DR and PIRA as well as

Fig. 2

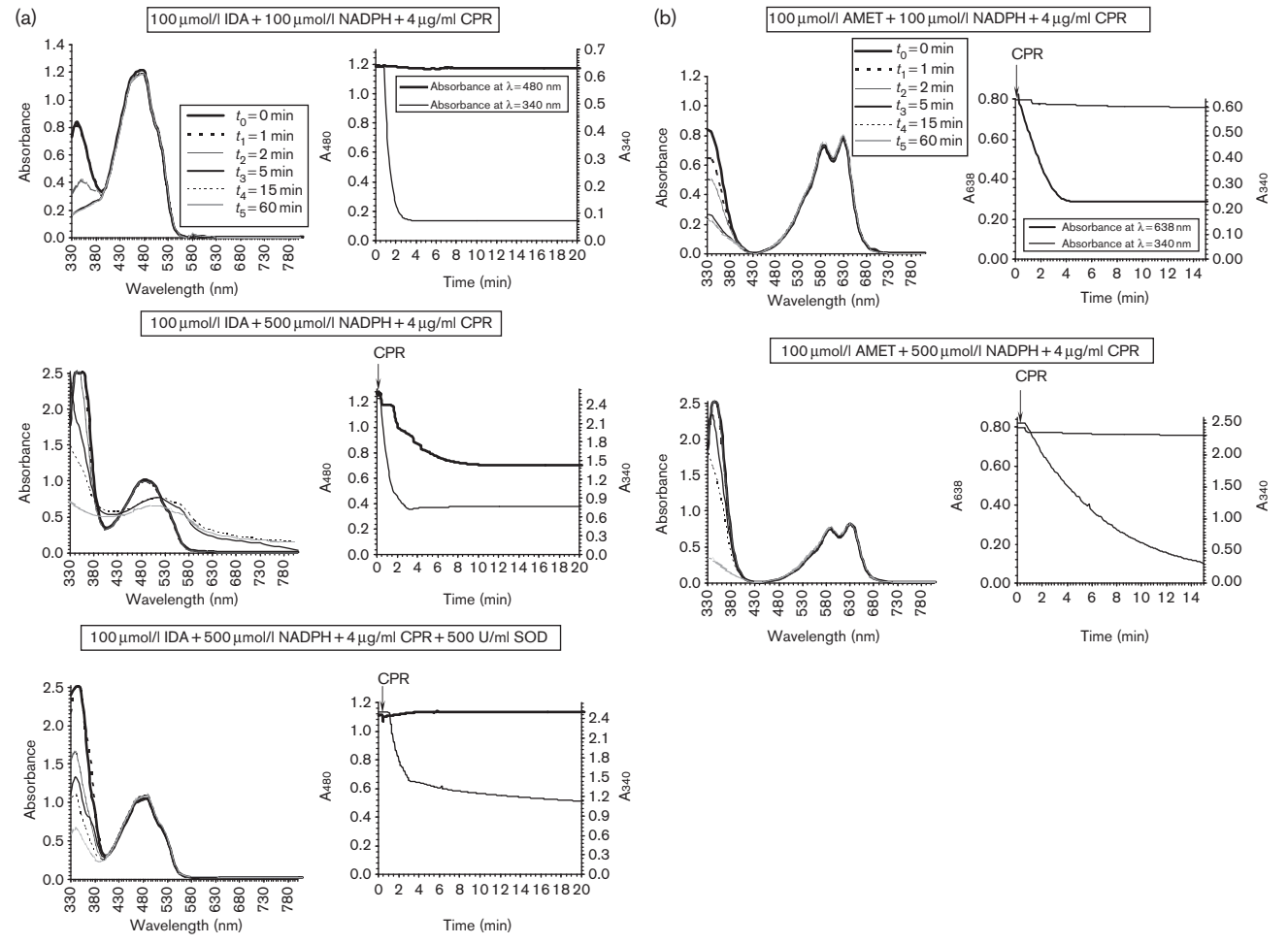
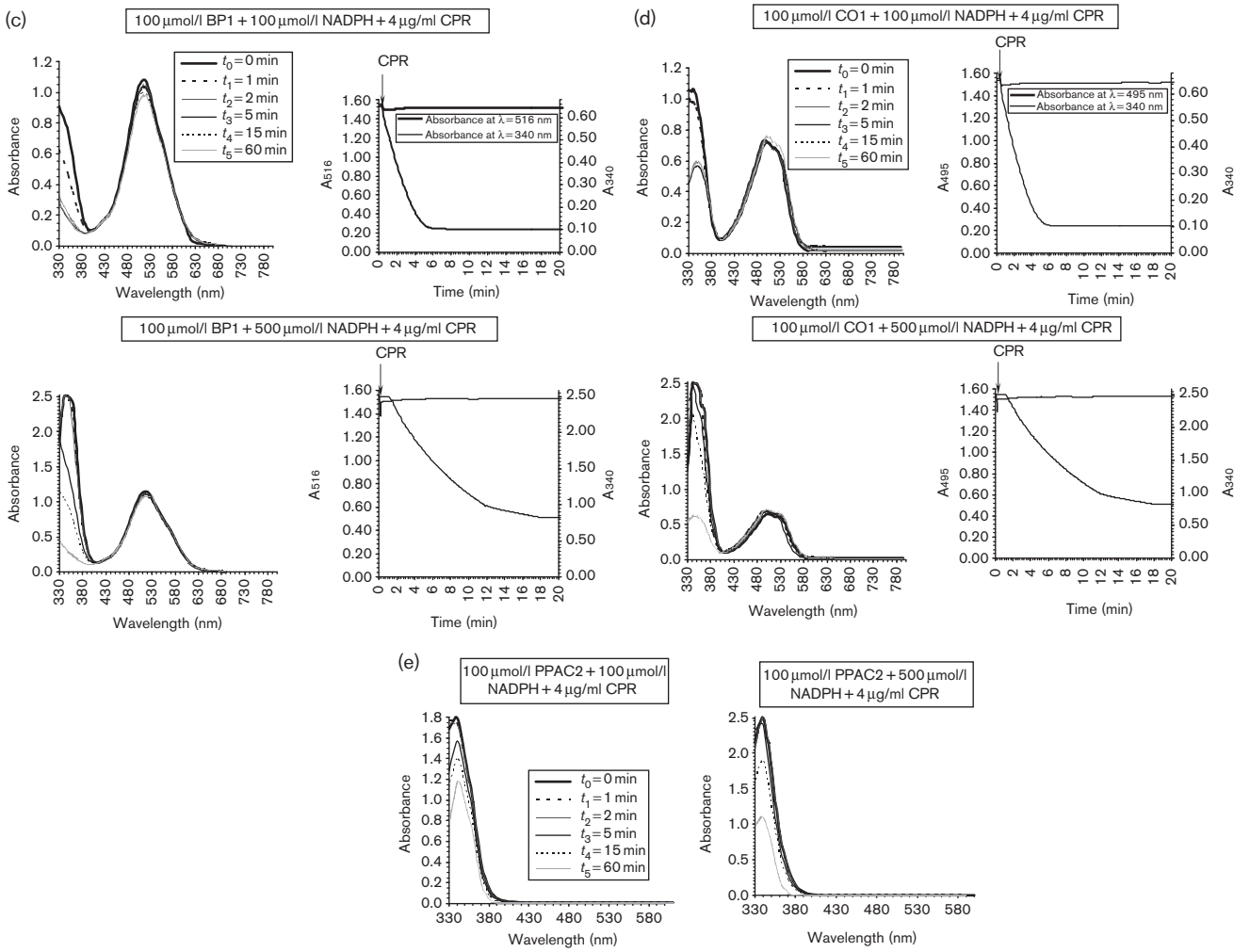


Fig. 2 (*continued*)



Spectroscopic changes followed during incubation of examined compounds (a) idarubicin (IDA), (b) ametantrone (AMET), (c) benzoperimidine (BP1), (d) anthrapyridone (CO1) and (e) pyrazolopyrimidoacridine (PPAC2) in enzymatic systems. The selected absorption wavelengths represent the maximum absorption wavelengths for NADPH and examined compounds. The samples contained 100 µmol/l compound, the indicated amount of NADPH (100 or 500 µmol/l, respectively), 4 µg/ml cytochrome P450 reductase (CPR) and the indicated amount of superoxide dismutase (SOD) (0 or 500 U/ml, respectively). The measurements were carried out in 0.01 mol/l K_2HPO_4/KH_2PO_4 buffer (pH 7.25) at 37°C. The reactions were initiated by the addition of CPR. Data shown are from a representative experiment.

anthracenedione: MX) [14–16]. However, in the case of other studied compounds (BP1, CO1 and PPAC2), it was found that the incubation of these quinone-modified agents with CPR at a high NADPH concentration (500 µmol/l) did not cause any changes in the cytotoxic effect on HL60 sensitive and resistant (HL60/VINC and HL60/DOX) cells in comparison with the compound alone (Fig. 3c–e).

IC_{50} values (drug concentrations required to inhibit 50% of cell growth) are summarized in Table 2. As can be seen, studied compounds in the nonactivated forms exhibited very different cytotoxic activity against sensitive HL60 and MDR (HL60/VINC and HL60/DOX) cells. A significant decrease in the IC_{50} values was observed for IDA and AMET activated by CPR in comparison with the drug alone (nonactivated) not only for sensitive HL60 but also in the case of resistant (HL60/VINC and HL60/DOX) cells examined. In contrast, in the case of the quinone-modified agents studied (BP1, CO1 and PPAC2), no statistically significant changes were observed between IC_{50} values found for a compound alone and incubated in the presence of the activating system for all studied cells (HL60, HL60/VINC and HL60/DOX).

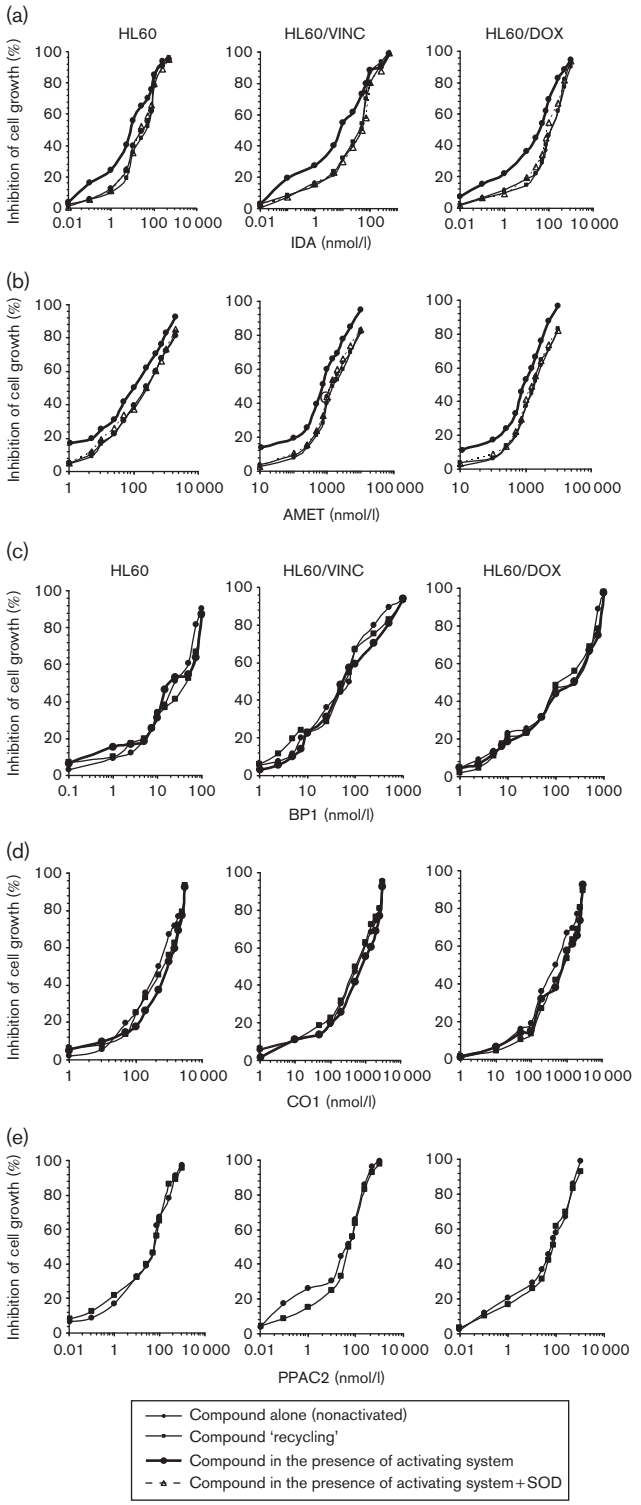
Interaction of examined compounds with intact cells

The interaction of examined compounds with intact HL60, HL60/VINC and HL60/DOX cells was studied for the reference compound IDA (1 µmol/l) and two selected quinone-modified compounds (BP1 and CO1; 1 µmol/l) with the high kinetics of cellular influx [20,26,27]. These studies were performed using our spectrofluorimetric

method largely described for anthracycline drugs as well as for novel groups of antitumour agents, among them benzoperimidines and anthrapyridones [20,26–30]. It allows continuous monitoring of the uptake of fluorescent molecules intercalating between the base pairs of nuclear DNA. As shown in Fig. 4a, in the case of the reference compound IDA, during the incubation time of the drug alone (1 μmol/l) with examined cells (HL60, HL60/VINC and HL60/DOX), a significant decrease in the fluorescence signal was observed until the steady state was reached. Similar results were found for this compound operating in the redox cycling (at 100 μmol/l NADPH concentration) during the incubation with sensitive (HL60) as well as resistant (HL60/VINC and HL60/DOX) cells. In contrast, no decrease in the fluorescence signal was observed for 1 μmol/l IDA undergoing reductive conversion (in the presence of an activating system, at 500 μmol/l NADPH concentration), indicating another type of interaction of generated IDA metabolites with intact cells (no intercalation between the base pairs of nuclear DNA). Furthermore, it should be emphasized that in the presence of SOD (500 U/ml) in the activating system, the interaction of 1 μmol/l IDA with cells was very similar to the interaction observed in the case of the drug alone or operating in the redox cycling, confirming that the decomposition of $O_2^{\bullet}$ abolishes its reductive activation by CPR.

In the case of quinone-modified compounds (BP1 and CO1) during the incubation time of the compound alone (1 μmol/l) with examined cells (HL60, HL60/VINC and HL60/DOX), a significant decrease in the fluorescence signal was observed until the steady state was reached. Furthermore, in contrast to the results found for IDA, similar uptake curves were recorded for those compounds operating in the redox cycling (at 100 μmol/l NADPH concentration) as well as pretreated with CPR at a high NADPH concentration (500 μmol/l) in the case of sensitive (HL60) as well as resistant (HL60/VINC and HL60/DOX) cells (Fig. 4b–c).

Fig. 3



Cytotoxic activity of examined compounds (a) idarubicin (IDA), (b) ametantrone (AMET), (c) benzoperimidine (BP1), (d) anthrapyridone (CO1) and (e) pyrazolopyrimidoacridine (PPAC2) towards HL60, HL60/VINC and HL60/DOX cells. The enzymatic samples contained for 'compound recycling': 100 μmol/l compound, 100 μmol/l NADPH and 4 μg/ml cytochrome P450 reductase (CPR); for the compound in the presence of the activating system: 100 μmol/l drug, 500 μmol/l NADPH and 4 μg/ml CPR; for the compound in the presence of the activating system + superoxide dismutase (SOD): 100 μmol/l compound, 500 μmol/l NADPH, 4 μg/ml CPR and 500 U/ml SOD (0.01 mol/l K_2HPO_4/KH_2PO_4 buffer, pH 7.25; 37°C). The appropriate volumes of the enzymatic samples were added directly to the cell suspensions to yield 0.01–500 nmol/l (IDA), 0.1 nmol/l–10 μmol/l (AMET), 0.1 nmol/l–1 μmol/l (BP1), 0.1 nmol/l–3 μmol/l (CO1) and 0.01 nmol/l–1 μmol/l (PPAC2), respectively, for all tested cells. The cytotoxic effect of the studied agents was determined by incubating cells (10^5) with 10 different concentrations of the compound for 72 h. The data points are from a representative experiment.

Intracellular accumulation of examined compounds in HL60, HL60/VINC and HL60/DOX cells

The intracellular accumulation of examined compounds IDA, AMET, BP1 and CO1 alone (nonactivated), acting

Table 2 Cytotoxic activity of examined compounds towards the HL60 cell line and its multidrug-resistant sublines: HL60/VINC and HL60/DOX

	Cell line		
	IC_{50} (nmol/l)		
Compound	HL60	HL60/VINC	HL60/DOX
IDA (alone, nonactivated)	24.0 ± 4.0	42.2 ± 6.0	119 ± 35
IDA (in the presence of an activating system)	9.5 ± 1.5**	17.7 ± 4.7**	31.9 ± 7.0*
AMET (alone, nonactivated)	211 ± 28	1262 ± 102	1960 ± 198
AMET (in the presence of an activating system)	110 ± 36**	627 ± 63***	1057 ± 84**
BP1 (alone, nonactivated)	11.5 ± 3.8	37.7 ± 9.2	133 ± 30
BP1 (in the presence of an activating system)	11.4 ± 1.9*	36.0 ± 7.1	135 ± 18
CO1 (alone, nonactivated)	378 ± 74	530 ± 108	552 ± 118
CO1 (in the presence of an activating system)	362 ± 92	518 ± 103	547 ± 35
PPAC2 (alone, nonactivated)	31.3 ± 3.7	38.9 ± 8.8	66.0 ± 8.5
PPAC2 (in the presence of an activating system)	31.5 ± 3.3	39.4 ± 9.1	63.0 ± 5.9

IC_{50} is the drug concentration required to inhibit 50% of cell growth. The values represent mean ± SD of at least five independent experiments.
AMET, ametantrone; BP1, benzoperimidine; CO1, anthrapyridone; IDA, idarubicin; PPAC2, pyrazolopyrimidoacridine.
The significance level of the differences observed (Student's *t*-test): *$P<0.01$; **$P<0.001$; ***$P<0.0001$ vs. values found for the drug alone (nonactivated).

in the redox cycling and incubating in the presence of the activating system (without or with the addition of SOD), was determined by flow cytometry. Figure 5 shows the histograms obtained in typical experiments for examined samples after 15 min of incubation of cells with compounds with high kinetics of cellular uptake (IDA, BP1 and CO1) or 5 h of incubation with a compound with a low kinetics of cellular uptake (AMET), respectively. The results obtained after the incubation of sensitive HL60 as well as resistant (HL60/VINC and HL60/DOX) cells with agents with a nonmodified quinone structure (IDA and AMET; 1 μmol/l) pretreated with CPR at a high NADPH concentration (500 μmol/l) showed a significant increase in intracellular drug fluorescence in comparison with the level found for the drug alone (nonactivated). In contrast, it was found that the intracellular fluorescence of these drugs operating in the redox cycling (at low NADPH concentration) as well as preincubated in the presence of the activating system containing SOD were similar to the fluorescence levels observed for drugs alone (Fig. 5a–b). Interestingly, it was found for all examined cells (HL60, HL60/VINC and HL60/DOX) that the intracellular fluorescence level of quinone-modified compounds (BP1 and CO1) operating in the redox cycling as well as preincubated in the presence of the activating system was comparable with the intracellular fluorescence level observed for these compounds alone (Fig. 5c–d).

Interaction of examined compounds with naked DNA

The interaction of selected compounds IDA, BP1 and CO1 (1 μmol/l) with naked DNA was studied by fluorimetric titration with naked DNA at 37°C in HEPES buffer (pH 7.25). A significant quenching of the fluorescence signal for all studied agents was observed in the case of compounds alone (nonactivated) and operating in the redox cycling after the addition of naked DNA (Fig. 6). However, a much less significant decrease in the fluorescence signal was observed for IDA undergoing reductive conversion whereas in the presence of SOD (500 U/ml) in the activating system, the interaction of this agent with naked DNA was very similar to the interaction observed in the case of the drug alone or operating in the redox cycling (Fig. 6a). In contrast, the results of our studies carried out under the same conditions for 1 μmol/l BP1 and 1 μmol/l CO1 showed that the preincubation of these quinone-modified compounds in the presence of the activating system did not alter their interaction with naked DNA in comparison with the compound alone or operating in the redox cycling (Fig. 6b–c).

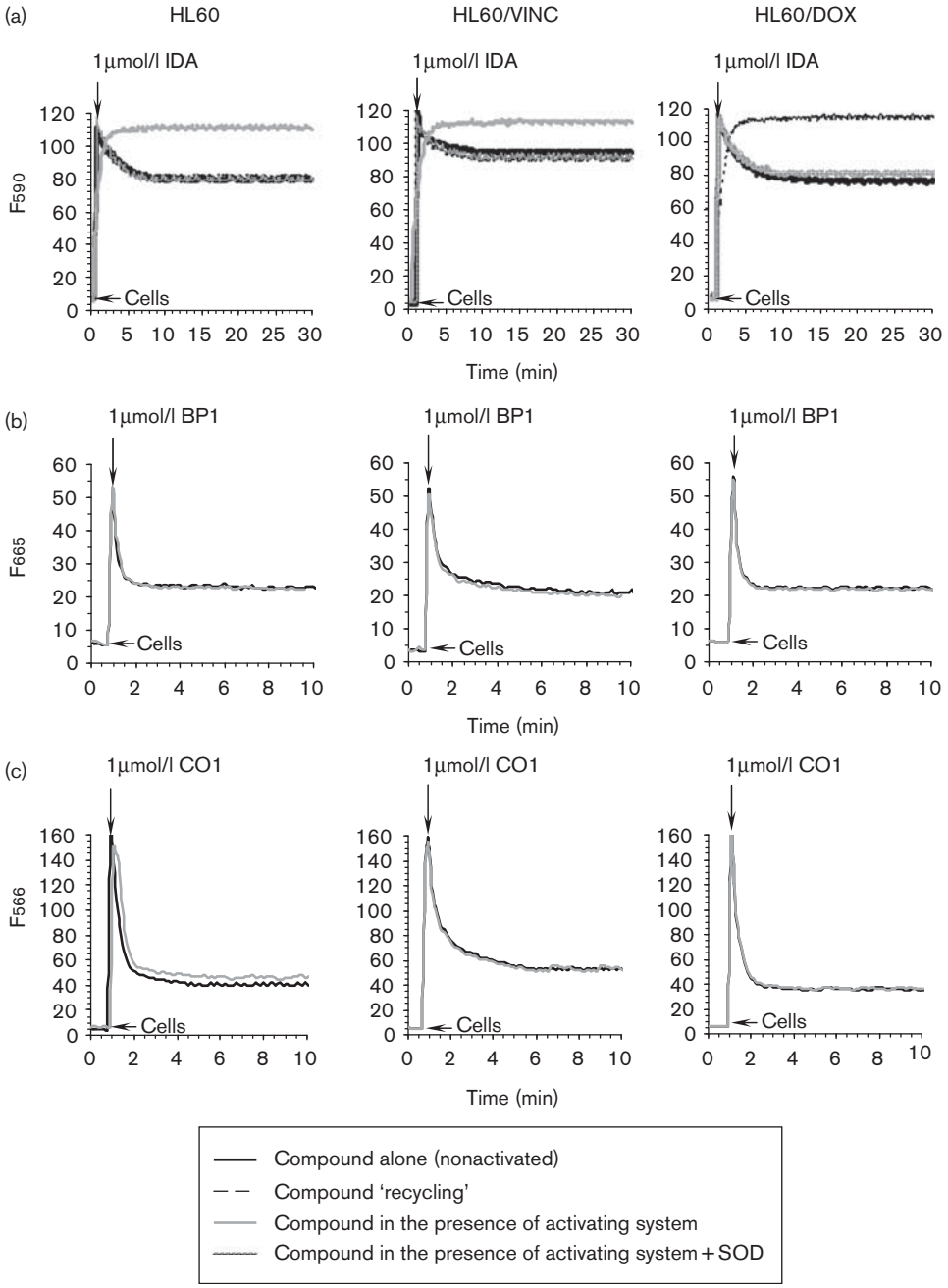
Discussion

There is an increasing body of evidence indicating that the reductive activation of antitumour drugs could lead to a significant increase in the cytotoxic activity of these compounds against tumour cells [6,7,10–13].

Our previous results found for the anthracycline drug DOX and the anthracenedione agent MX proved that these antitumour compounds belonging to the anthraquinone family could undergo bioreductive activation by CPR and that this activation has a high impact on increasing their activity not only with regard to the sensitive leukaemia HL60 cell line but also against its MDR sublines overexpressing P-glycoprotein (HL60/VINC) and MRP1 (HL60/DOX) [14,15]. Moreover, in our recent study aimed at elucidating the role of structural factors of antitumour anthracycline drugs in the ability to undergo CPR-dependent reductive activation, it was observed that only anthracycline agents with a nonmodified quinone structure were susceptible to undergo a multistage chemical transformation, suggesting that the presence of a modified quinone function is the structural factor excluding the reductive activation of anthracycline compounds by CPR [17].

To verify and generalize our supposition, in the present study, we examined the ability of several antitumour

Fig. 4



Cellular uptake of examined compounds: (a) idarubicin (IDA), (b) benzoperimidine (BP1), (c) anthrapyridone (CO1) by HL60, HL60/VINC and HL60/DOX cells. Cells in the logarithmic growth phase were suspended in a cuvette filled with 2 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, 37°C) under vigorous stirring. At t_0, 20 μl of a drug solution (for 'compound-alone' assays) or 20 μl of an appropriate enzymatic sample (described in detail in Fig. 3) was added to the cell suspension, yielding a 1 μmol/l compound (IDA, BP1 or CO1, respectively) concentration. Fluorescence intensity at 590 nm for IDA, 665 nm for BP1 and 566 nm for CO1, respectively, was recorded as a function of incubation time until the steady state. The presented curves are from a representative experiment. SOD, superoxide dismutase.

anthraquinone derivatives and analogues to undergo bioreductive activation by CPR and determined the impact of this activation on increasing the activity against the human promyelocytic sensitive leukaemia HL60 cell line as well as its MDR sublines (HL60/VINC and HL60/DOX). The selected compounds belong to various chemical groups of bioreductive antitumour agents: anthracenediones (AMET), benzoperimidines (BP1), anthrapyridones (CO1) and pyrazolopyrimidoacridines (PPAC2) (Fig. 1). It was found that the studied

compounds in the nonactivated forms exhibited very different cytotoxic activity against sensitive HL60 as well as MDR (HL60/VINC and HL60/DOX) cells and there is no simple relationship between the presence/absence of the nonmodified quinone structure and the cytotoxic activity of examined anthraquinone derivatives and analogues against sensitive HL60 and MDR (HL60/VINC and HL60/DOX) cells.

Subsequently, it was shown that at a high NADPH concentration (500 µmol/l), besides the reference compound, IDA, only AMET with an unmodified quinone structure was susceptible to O_2•-dependent reductive activation by CPR, but contrary to the results found for IDA and other anthracycline drugs (DOX, PIRA), enzymatic activation of AMET occurred without modifications in its absorption spectra, showing that the formation of reactive metabolites of AMET did not occur with the participation of the chromophore part. Similar results were previously found for another anthracenedione drug, MX [15]. This suggests that the modifications of compounds belonging to this chemical group occurred in their side chains containing reactive secondary aminoalkyl groups. The involvement of aminoalkyl groups was also proposed for the formation of DNA adducts by formaldehyde-activated MX [31].

Fig. 5

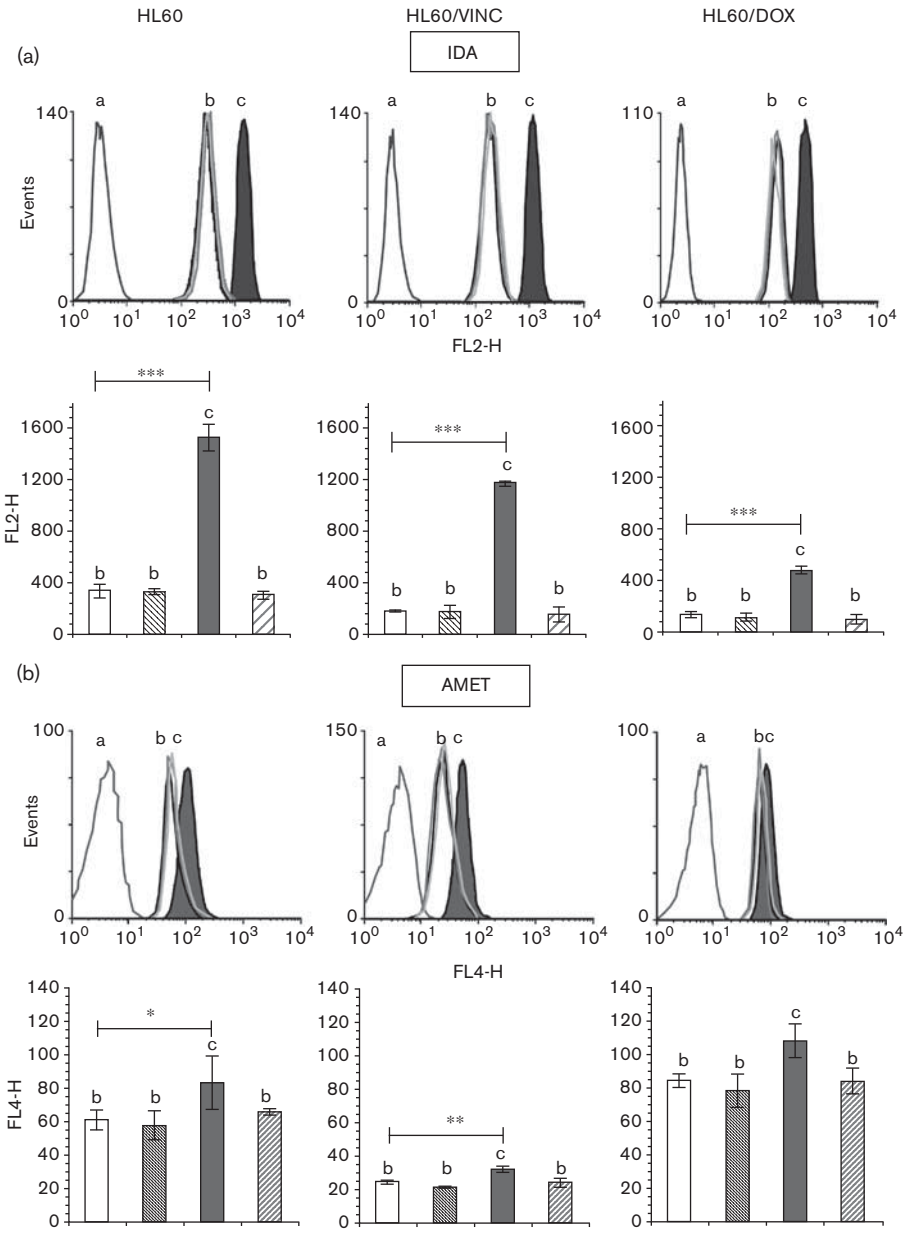
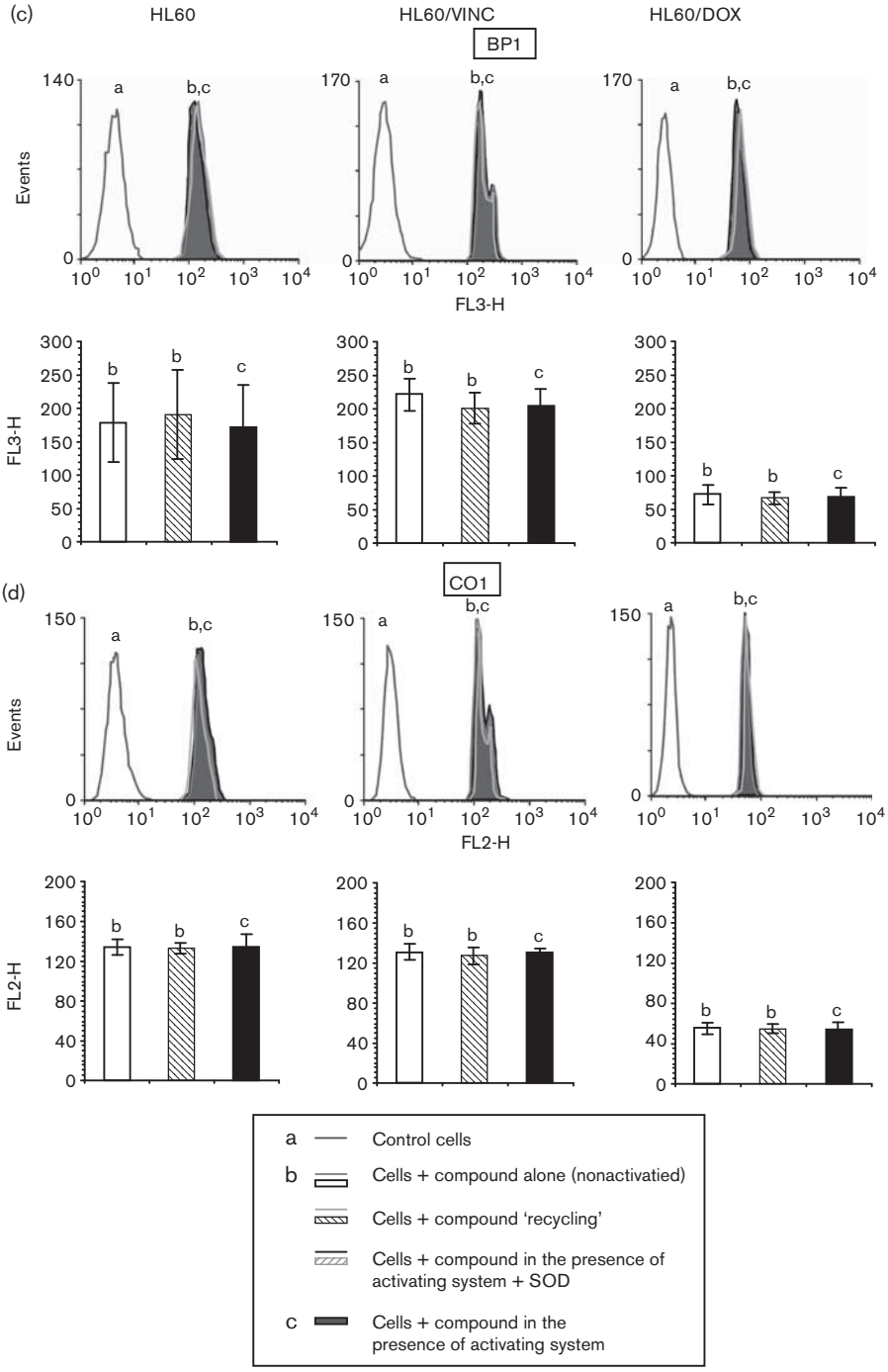
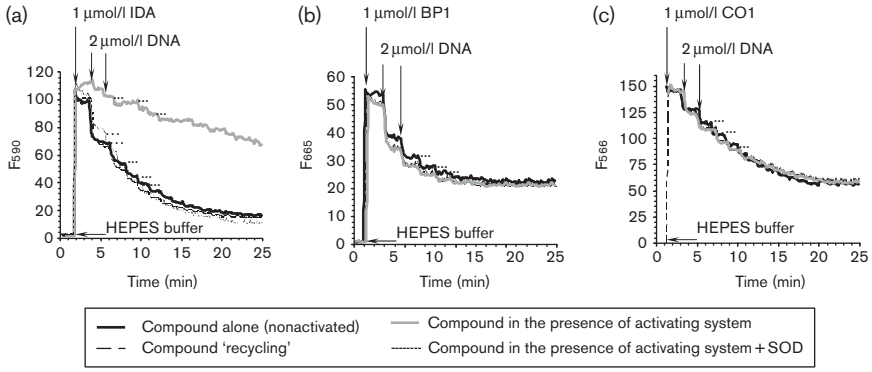


Fig. 5 (*continued*)



Intracellular accumulation of examined compounds (a) idarubicin (IDA), (b) ametantrone (AMET), (c) benzoperimidine (BP1), (d) anthrapyridone (CO1) in HL60, HL60/VINC and HL60/DOX cells. Cells in the 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, 37°C) to a final concentration of 10^6 cells/ml. At t_0, 10 µl of a compound solution (for 'compound-alone' assays) or 10 µl of an appropriate enzymatic sample (described in detail in Fig. 3) was added to the cell suspension, yielding 1 µmol/l compound (IDA, AMET, BP1 or CO1, respectively) concentration, and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 15 min in the case of IDA, BP1 and CO1 or 5 h in the case of AMET, respectively. After the indicated incubation time, the intensity of the fluorescence signal was measured within the FL-2 fluorescence channel (bandpass filter, $\lambda = 585 \pm 21$ nm) for IDA and CO1 or within the FL-3 fluorescence channel (longpass filter, $\lambda \geq 670$ nm) in the case of BP1 after an excitation with the argon-ion laser ($\lambda_{ex} = 488$ nm) or within the FL-4 fluorescence channel (bandpass filter, $\lambda = 661 \pm 8$ nm) after an excitation with the red-diode laser ($\lambda_{ex} = 635$ nm) for AMET, respectively. For each experimental point, the fluorescence signal of 1×10^4 cells was measured. The experiment was repeated five times and the presented histograms are representative examples. The significance level of the differences observed (Student's *t*-test): $^*P < 0.01$; $^{**}P < 0.001$ $^{***}P < 0.0001$ vs. values found for the compound alone (nonactivated). SOD, superoxide dismutase.

Fig. 6



Interaction of examined anthracycline drugs: (a) idarubicin (IDA), (b) benzoperimidine (BP1), (c) anthrapyridone (CO1) with naked DNA. At t_0 20 μl of a compound solution (for 'compound alone' assays) or 20 μl of an appropriate enzymatic sample (described in detail in the legend to Fig. 3) was added to 2 ml 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, 37°C), yielding 1 μmol/l compound (IDA, BP1 or CO1, respectively) concentration. The binding of examined compounds to DNA (0–50 μmol/l) was followed by monitoring the decrease in the fluorescence signal at 590 nm for IDA, 665 nm for BP1 and 566 nm for CO1, respectively, after the addition of 2 μmol/l DNA portions. Data shown are from a representative experiment. SOD, superoxide dismutase.

Furthermore, it was confirmed that CPR-dependent activation of AMET led to significant augmentation in its intracellular accumulation and had a huge impact on increasing the activity with regard to the sensitive leukaemia HL60 cell line as well as against its MDR sublines (HL60/VINC and HL60/DOX), whereas the presence of CPR catalysing only the redox cycling of this compounds did not exert any effect on its activity against studied leukaemia cells. It is worth noting that the data presented for AMET (comparable increase of about two-fold in cytotoxic activity against resistant HL60/VINC and HL60/DOX cells as was observed in the case of the sensitive HL60 cell line), were similar to the results found previously for anthracyclines with an unmodified quinone structure: DR, IDA, PIRA and anthracenedione drug MX [14,15,17]. The obtained results suggest that reactive metabolites of AMET generated extracellulary are able to enter the cell and bind to cellular targets before being extruded by MDR exporting pumps.

In contrast, convincing results found for other studied agents with a modified quinone structure (benzoperimidine BP1, anthrapyridone CO1 and pyrazolopyrimidoacridine PPAC2) show that they were not able to undergo reductive activation by CPR. No changes were observed in the absorption spectra of these compounds in the presence of the activating system and no effect of exogenously added CPR was observed in modulating their cytotoxicity against sensitive HL60 as well as resistant HL60/VINC and HL60/DOX cells in comparison with the compound alone. Furthermore, no increase in the intracellular accumulation of these agents and no changes in their interaction with intact cells and in binding to naked DNA in the presence of the activating system were observed, additionally indicating that examined anthraquinone analogues with a modified quinone structure are not susceptible to reductive conversion by CPR.

In conclusion, the data presented in the present study for bioreductive anthraquinone derivatives and analogues belonging to various chemical groups, together with the results reported recently for antitumour anthracycline drugs and the anthracenedione agent MX, strongly suggest that the presence of an unmodified quinone grouping is the structural factor determining the ability of bioreductive agents to undergo CPR-dependent reductive activation with a high impact on increasing their activity not only towards sensitive leukaemia cells but also against resistant cells overexpressing MDR-exporting pumps. However, further studies are needed to determine whether CPR-dependent activation of the studied compounds inhibits only their efflux by MDR/MRP transporters or also influences cellular downstream pathways. These studies are in progress in our laboratory.

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Conflicts of interest

There are no conflicts of interest.

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