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# The ability of selected pyridinium salts to increase the cytotoxic activity of vincristine but not doxorubicin towards sensitive and multidrug resistant promyelocytic leukaemia HL60 cells

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## Abstract

The aim of this study was to examine the effect of selected pyridinium salts, 1-methyl-3-nitropyridine chloride ($MNP^+Cl^-$) and 3,3,6,6,10-pentamethyl-3,4,6,7-tetrahydro-[1,8(2*H*,5*H*)-dion]acridine chloride ($MDION^+Cl^-$), on the activity of doxorubicin (DOX) and vincristine (VINC) towards human promyelocytic leukaemia HL60 cells as well as its multidrug resistant (MDR) sublines exhibiting two different phenotypes of MDR related to the overexpression of P-glycoprotein (HL60/VINC) or MRP1 (HL60/DOX). MNP and MDION salts were much less cytotoxic themselves (about 100-fold and 2000-fold compared with DOX and VINC, respectively) against HL60 cells but, in contrast to DOX and VINC, they conserved an important cytotoxic activity towards resistant HL60/VINC and HL60/DOX cells (resistance factor, RF = 2 – 4.5). It was shown that $MNP^+Cl^-$ and $MDION^+Cl^-$ increased the cytotoxicity of non-bioreductive antitumour agent VINC towards human promyelocytic leukaemia HL60 cells and its resistant sublines HL60/VINC and HL60/DOX. However, in the case of DOX the decrease in its cytotoxic activity towards all studied cell lines was observed in the presence of $MNP^+Cl^-$ and $MDION^+Cl^-$. Presented data suggest that the bioreductive drug DOX, in contrast to VINC, could compete with pyridinium salts ($MNP^+Cl^-$ and $MDION^+Cl^-$) for NADPH-dependent oxidoreductases and for undergoing cellular reductive activation. This could explain the inefficiency of these salts to increase the cytotoxic activity of DOX against examined leukaemic HL60 cell line and its MDR sublines, HL60/VINC and HL60/DOX.

## Introduction

The anthracycline anti-tumour agent doxorubicin and vinca alkaloid vincristine (Figure 1A) are among the most effective drugs currently available for the treatment of various human neoplastic diseases. Doxorubicin is largely employed in the treatment of lymphomas, sarcomas and carcinomas, including breast cancers (Lown 1993; Rivera 2003). Different mechanisms have been proposed for the anti-tumour effects of doxorubicin, including DNA intercalation with consequent inhibition of DNA biosynthesis, alkylation of DNA and DNA cross-linking, inhibition of topoisomerase II, activation of signalling pathways and apoptosis (Gewirtz 1999; Minotti et al 2004). Additionally, doxorubicin is a well-known bioreductive anti-tumour drug. Its ability to undergo a one-electron reduction by NADP(H)-dependent cellular oxidoreductases is related to the formation of an unstable semiquionone radical followed by the production of reactive oxygen species and induction of DNA damage (Mordente et al 2001; Pawlowska et al 2003). Vincristine is used mainly in the treatment of acute lymphoblastic leukaemia and Hodgkin's disease (Checchi et al 2003; Hadfield et al 2003). The anti-tumour activity of this drug is associated with binding to tubulin heterodimers causing microtubule depolymerization and blocking of cell division at the metaphase/anaphase junction of mitosis (Kavallaris et al 2001; Drukman & Kavallaris 2002; Jordan 2002; Verrills et al 2003). There are many data in the literature indicating that vincristine induces apoptosis in tumour cells (Groninger et al 2002; Huang et al 2004). It has also been reported that this drug inhibits

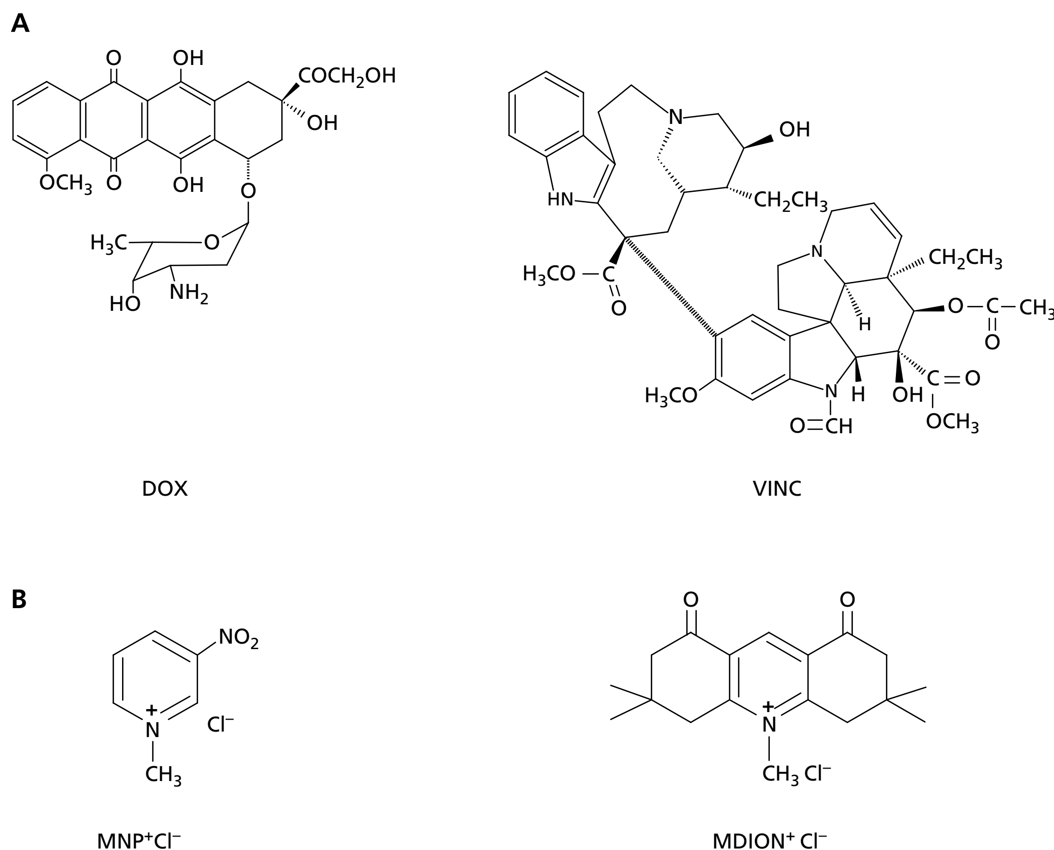


**Figure 1** Structure of examined compounds—anti-tumour drugs, doxorubicin (DOX) and vincristine (VINC) (A), and pyridinium salts, 1-methyl-3-nitropyridine chloride ($MNP^+Cl^-$) and 3,3,6,6,10-pentamethyl-3,4,6,7-tetrahydro-[1,8(2*H*,5*H*)-dion]acridine chloride ($MDION^+Cl^-$) (B).

biosynthesis of proteins and nucleic acids as well as altering lipid metabolism (Beck et al 2003).

The clinical usefulness of doxorubicin, vincristine and other anthracycline and vinca alkaloid drugs is limited by the occurrence of multidrug resistance (MDR) associated with the overexpression of membrane transporters (e.g. P-glycoprotein, MRP1 BCRP/MXR1) belonging to the ATP-binding cassette protein family (Chaudhary & Roninson 1993; Borst et al 2000; Gottesman 2002; Longley & Johnston 2005). These transporters are responsible for the active ATP-dependent efflux of drugs out of resistant cells resulting in a decreased intracellular accumulation insufficient to inhibit resistant cell proliferation (Zaman et al 1994; Paul et al 1996; Ejendal & Hrycyna 2002). Therefore, much effort is focused on the search for agents able to increase the activity of anthracycline and vinca alkaloid drugs (Gtowacka-Rogacka et al 2004; Szakācs et al 2006).

Recently, some interest has been paid to the biological activity of compounds capable of shifting $NAD(P)H/NAD(P)^+$ equilibrium in non-enzymatic and enzyme-mediated processes (Ogata et al 1998, 2000, 2002; Wieczorkowska et al 2003, 2007; Olek et al 2004). The changes in NAD(P)H and $NAD(P)^+$ concentrations can disturb the cellular metabolism by affecting the rates of NAD(P)H- and $NAD(P)^+$-dependent reactions (Crowley et al 2000, Liu et al 2001; Lavilla et al 2002; Lin & Guarente 2003). Consequently, the cells with impaired metabolism should be more sensitive to cytotoxic agents. The aim of this study was to examine the effect of selected pyridinium salts (Figure 1B) on the activity of doxorubicin and vincristine towards human promyelocytic leukaemia HL60 cells 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).

## Materials and Methods

### Chemicals

NADPH and vincristine were obtained from Sigma Chemical Co. (St Louis, MO). Doxorubicin was kindly provided by Pharmacia-Upjohn (Milan, Italy). 1-Methyl-3-nitropyridine chloride ($MNP^+Cl^-$) and 3,3,6,6,10-pentamethyl-3,4,6,7-tetrahydro-[1,8(2*H*,5*H*)-dion]acridine chloride ($MDION^+Cl^-$) were synthesized according to the procedure published elsewhere (Srividja et al 1996; Marcinek et al 2000; Wieczorkowska et al 2003). Stock solutions ($C_0 = 10^{-3}$ M) of doxorubicin, vincristine, $MNP^+Cl^-$, $MDION^+Cl^-$ and NADPH were prepared just before use.

NADPH cytochrome P450 reductase (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 (Smith et al 1994).

### Cell culture

HL60 human promyelocytic leukaemia cell line (Division of Biology, Kansas State University, Manhattan, KS 66506) and its resistant sublines: HL60/VINC (overexpressing P-glycoprotein) (McGrath et al 1989) and HL60/DOX (overexpressing MRP1) (Marsh et al 1986; Krishnamachary & Center 1993) were cultured. The cells were grown in RPMI 1640 (Gibco Limited) medium supplemented with 2 mM glutamine and 10% fetal bovine serum (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 nM vincristine and HL60/DOX cells in the presence of 200 nM doxorubicin. All cultures (HL60, HL60/VINC, HL60/DOX) initiated at a density of $10^5$ cells/mL grew exponentially to about $10^6$ cells/mL in 72 h. They were counted before the assay using a Burker haemocytometer. Cell viability was assessed by trypan blue exclusion test.

### Cell growth inhibition

For each cell line, the effect of doxorubicin or vincristine, respectively in the absence and in the presence of pyridinium salts ($MNP^+Cl^-$ and $MDION^+Cl^-$), on the cell growth was determined by incubating cells ($10^5$/mL) with 10 different concentrations of the compound for 72 h in standard 96-well plates. Depending on the cell line studied, HL60, HL60/VINC or HL60/DOX cells, the concentrations varied for doxorubicin in the ranges: 0.1 nM to 1 $\mu$M, 1 nM to 5 $\mu$M and 10 nM to 10 $\mu$M; for vincristine: 0.001–100 nM, 1 nM to 3 $\mu$M and 0.1–200 nM; for $MNP^+Cl^-$: 0.1–10 $\mu$M, 1–20 $\mu$M, 1–50 $\mu$M and for $MDION^+Cl^-$: 0.1–10 $\mu$M, 1–30 $\mu$M, 1–100 $\mu$M, respectively. The values of compound concentration (doxorubicin, vincristine, $MNP^+Cl^-$ and $MDION^+Cl^-$, respectively) required to inhibit by 50% (IC50) the cell growth was determined by counting the viable cells in the presence of trypan blue using a Burker hemocytometer. In calculating the percentage of cell growth inhibition, untreated cells were used as the control.

### Spectroscopic studies

The reaction mixtures in 0.01 M $K_2HPO_4/KH_2PO_4$ buffer (pH 7.25) contained: 100 $\mu$M $MNP^+Cl^-$ or $MDION^+Cl^-$, 100 $\mu$M NADPH, 0 or 100 $\mu$M doxorubicin, 0 or 100 $\mu$M vincristine and 0 or 10 $\mu g\,mL^{-1}$ CPR. All the reactions were initiated by the addition of NADPH and conducted at 37°C. Absorption spectra 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 $\varepsilon = 6220\,M^{-1}cm^{-1}$. Absorption measurements were made on a Marcel E330 spectrophotometer.

### Statistical analysis

Results are presented as the mean ± s.d. of four independent experiments. Statistical analysis of the significance level of the differences observed between values of the percentage of cell growth inhibition found for doxorubicin or vincristine, respectively, in the absence vs values found in the presence of pyridinium salts ($MNP^+Cl^-$ and $MDION^+Cl^-$) was carried out using parametric analysis of variance and post-hoc RIR Tukey's test. $P < 0.05$ was considered as a significant difference.

## Results and Discussion

### Effect of selected pyridinium salts ($MNP^+Cl^-$ and $MDION^+Cl^-$) on the cytotoxic activity of doxorubicin and vincristine towards sensitive (HL60) and resistant (HL60/VINC and HL60/DOX) human promyelocytic leukaemia cells

The ability of doxorubicin and vincristine to inhibit the growth of 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 pyridinium salts ($MNP^+Cl^-$ and $MDION^+Cl^-$). 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). IC50 values (compound concentration required to inhibit the cell growth by 50%) for all studied agents are summarized in Table 1. Doxorubicin and vincristine exhibited a high cytotoxic activity against human promyelocytic sensitive leukaemia HL60 cell line (IC50 = 45 ± 17 nM and IC50 = 1.81 ± 0.47 nM for doxorubicin and vincristine, respectively). However, an important decrease in their cytotoxic activity was observed against MDR sublines exhibiting two different phenotypes of MDR related to the overexpression of P-glycoprotein (HL60/VINC, resistance factor RF = 10 and RF = 889 for doxorubicin and vincristine, respectively) or

**Table 1** The ability of anti-tumour drugs, doxorubicin and vincristine, as well as pyridinium salts, $MNP^+Cl^-$ and $MDION^+Cl^-$ (used as single agents), to inhibit the growth of HL60 cell line and its multidrug resistant sublines, HL60/VINC and HL60/DOX

| Drug | HL60 IC50 ($\mu$M) | HL60/VINC IC50 ($\mu$M) | RF | HL60/DOX IC50 ($\mu$M) | RF |
|---|---|---|---|---|---|
| Doxorubicin | 0.045 ± 0.017 | 0.44 ± 0.13 | 10 | 1.64 ± 0.41 | 40 |
| Vincristine | 0.00181 ± 0.00047 | 1.59 ± 0.51 | 889 | 0.0325 ± 0.0082 | 18 |
| $MNP^+Cl^-$ | 3.49 ± 0.26 | 7.45 ± 0.23 | 2 | 9.26 ± 0.46 | 3 |
| $MDION^+Cl^-$ | 4.20 ± 0.83 | 11.21 ± 0.83 | 3 | 18.56 ± 0.67 | 4.5 |

IC50 is the compound concentration required to inhibit the cell growth by 50%. Resistance factor (RF) was calculated as RF = IC50(R)/IC50(S); S – sensitive HL60 cells, R – resistant HL60/VINC or HL60/DOX cells, respectively. The values represent mean ± s.d. of 4 independent determinations (made in triplicate).

MRP1 (HL60/DOX, RF = 40 and RF = 18 for doxorubicin and vincristine, respectively). MNP and MDION salts were much less cytotoxic themselves (about 100-fold and 2000-fold compared with doxorubicin and vincristine, respectively) against HL60 cells (IC50 = 3.49 ± 0.26 $\mu$M and IC50 = 4.20 ± 0.83 $\mu$M for $MNP^+Cl^-$ and $MDION^+Cl^-$, respectively) but, in contrast to doxorubicin and vincristine, they conserved an important cytotoxic activity towards resistant HL60/VINC and HL60/DOX cells (RF = 2 – 4.5).

The ability of pyridinium salts to modulate doxorubicin and vincristine cytotoxic activity has been determined at IC10 concentrations of salts (0.5 $\mu$M $MNP^+Cl^-$ and 0.5 $\mu$M $MDION^+Cl^-$ for sensitive HL60 cells; 1 $\mu$M $MNP^+Cl^-$ and 2 $\mu$M $MDION^+Cl^-$ in the case of resistant HL60/VINC and HL60/DOX cells) to avoid the high cytotoxic effects of pyridinium salts themselves. Moreover, it should be also noted that these concentrations seem to be clinically achievable. It was found that both $MNP^+Cl^-$ and $MDION^+Cl^-$ were able to increase the cytotoxic activity of vincristine against sensitive (HL60) and MDR resistant (HL60/VINC and HL60/DOX) cell lines ($P < 0.001$). This advantageous effect of $MNP^+Cl^-$ and $MDION^+Cl^-$ was observed over the wide range of vincristine concentrations (up to 10 nM, 1 $\mu$M and 100 nM in the case of HL60, HL60/VINC and HL60/DOX, respectively). However, in the case of doxorubicin a decrease in its cytotoxic activity towards all studied cell lines was observed in the presence of $MNP^+Cl^-$ and $MDION^+Cl^-$ ($P < 0.001$) (Figure 2).

### Effect of vincristine and doxorubicin on reductive properties of pyridinium salts ($MNP^+Cl^-$ and $MDION^+Cl^-$) related to their biological properties

It is proposed that the cytotoxic activity of the studied pyridinium salts against tumour cells is related to their ability to shift NAD(P)H/NAD(P)$^+$ equilibrium in non-enzymatic and enzyme-mediated processes and, in consequence, to disturb the cellular metabolism by affecting the rates of NAD(P) H- and NAD(P)$^+$-dependent reactions (Wieczorkowska et al 2003, 2007). It was demonstrated in this study that $MNP^+Cl^-$ and $MDION^+Cl^-$ increased the cytotoxicity of the non-bioreductive anti-tumour agent vincristine towards human

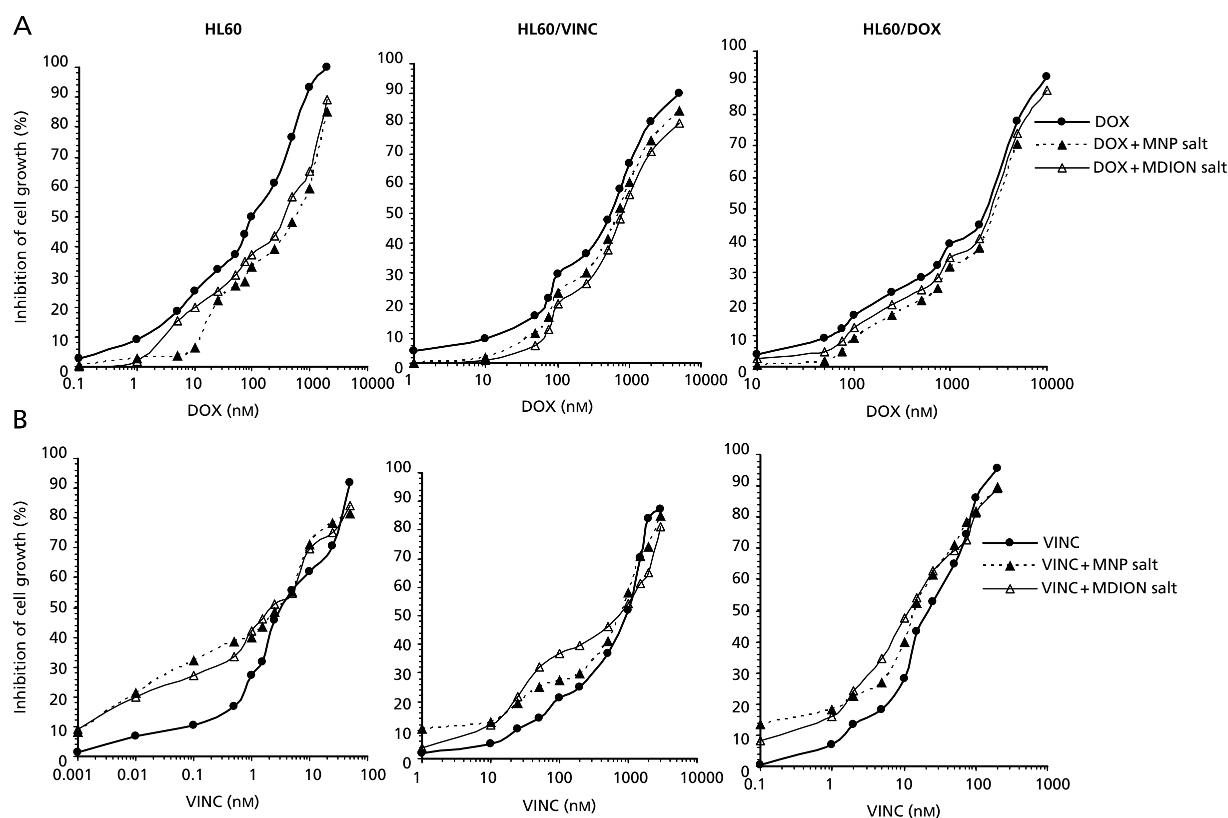


**Figure 2** The ability of doxorubicin (DOX; A) and vincristine (VINC; B) alone and in the presence of pyridinium salts ($MNP^+Cl^-$ and $MDION^+Cl^-$) to inhibit the growth of sensitive (HL60) and resistant (HL60/VINC and HL60/DOX) human promyelocytic leukaemia cells. Cell growth was determined by incubating cells ($10^5$/mL) with 10 different concentrations of DOX or VINC, respectively, in the presence or absence of pyridinium salts for 72 h. Pyridinium salts were used at IC10 concentrations (0.5 $\mu$M $MNP^+Cl^-$ and 0.5 $\mu$M $MDION^+Cl^-$ for sensitive HL60 cells; 1 $\mu$M $MNP^+Cl^-$ and 2 $\mu$M $MDION^+Cl^-$ in the case of resistant HL60/VINC and HL60/DOX cells). For each presented point the effect of pyridinium salts (ca. 10%) was subtracted. The data points are from a representative experiment. $P < 0.001$ (analysis of variance test), values of the percentage of cell growth inhibition found for DOX or VINC, respectively, in the absence vs values found in the presence of pyridinium salts ($MNP^+Cl^-$ and $MDION^+Cl^-$).

promyelocytic leukaemia HL60 cells and its resistant sublines HL60/VINC and HL60/DOX but they were unable to increase the cytotoxic activity of the bioreductive drug doxorubicin. Thus, it could be assumed that pyridinium salts compete with doxorubicin to undergo cellular reductive activation in NAD(P)H-dependent reactions. Therefore, in the second part of the work the effect of doxorubicin and vincristine on non-enzymatic and NADPH cytochrome P-450 reductase (CPR)-dependent reduction of $MNP^+Cl^-$ and $MDION^+Cl^-$ was studied using a spectrophotometric method. CPR is considered to be one of the most important oxidoreductases responsible for the cellular activation of bioreductive compounds (Bartoszek & Wolf 1992; Mewes et al 1993; Patterson et al 1995; Bailey et al 2001; Bartoszek 2002; Kostrzewa-Nowak et al 2005). It was found that $MNP^+Cl^-$ and $MDION^+Cl^-$, in contrast to doxorubicin, were able to stimulate NADPH oxidation in a non-enzymatic system and that $MDION^+Cl^-$ was about 5-fold more efficient than $MNP^+Cl^-$ ($V_0 = 2.13 \pm 0.21\ \mu$M min$^{-1}$ and $10.4 \pm 1.0\ \mu$M min$^{-1}$ for $MNP^+Cl^-$ and $MDION^+Cl^-$, respectively). In the presence of CPR, all three agents ($MNP^+Cl^-$, $MDION^+Cl^-$ and doxorubicin) caused an important NADPH oxidation. It was found that $MNP^+Cl^-$ was about 1.5-fold more active ($V_0 = 51.5 \pm 5.1\ \mu$M min$^{-1}$) in stimulating NADPH oxidation in CPR system than doxorubicin ($V_0 = 36.2 \pm 2.9\ \mu$M min$^{-1}$) whereas the activity of $MDION^+Cl^-$ in stimulating this reaction was about 3-fold less ($V_0 = 11.36 \pm 0.89\ \mu$M min$^{-1}$) than that of doxorubicin. Vincristine was unable to stimulate NADPH oxidation in either the non-enzymatic or enzymatic (CPR-mediated) processes (data not presented). Simultaneously, the reduction of $MNP^+Cl^-$ and $MDION^+Cl^-$ was studied by following changes in the absorption spectra during incubation of pyridinium salts with NADPH in non-enzymatic and enzymatic (CPR-dependent) systems. Figure 3 illustrates the representative absorption spectra of samples

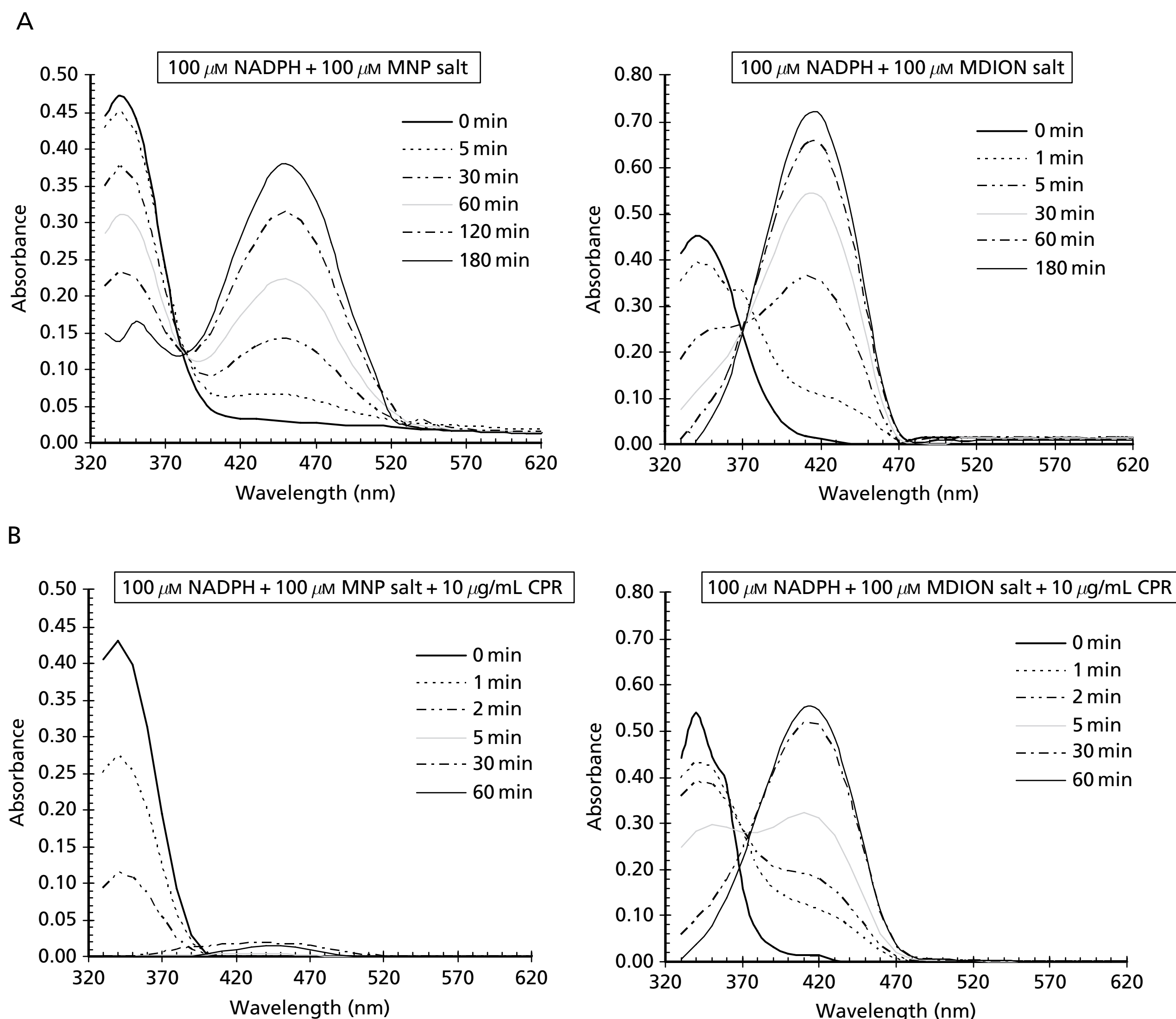


**Figure 3** Time-dependent changes in absorption spectra of equimolar (100 $\mu$M) mixture of NADPH and pyridinium salts ($MNP^+Cl^-$ and $MDION^+Cl^-$, respectively) in non-enzymatic (A) and enzymatic (CPR-mediated) processes (B). The measurements were carried out in 0.01 M $K_2HPO_4/KH_2PO_4$ buffer (pH 7.25) at 37°C. The reactions were initiated by the addition of NADPH. Data shown are from a representative experiment.

recorded during the incubation of 100 μM pyridinium salts ($MNP^+Cl^-$ or $MDION^+Cl^-$, respectively) with 100 μM NADPH in the absence (Figure 3A) and in the presence (Figure 3B) of 10 μg mL$^{-1}$ CPR. In the non-enzymatic system the decrease in $A_{340\,nm}$ due to NADPH oxidation and the appearance of absorption bands corresponding to the formation of the reduced forms of pyridinium salts were observed. The maximum absorption wavelengths for MNPH and MDIONH forms were 445 nm and 410 nm, respectively. Very similar results were found for the reduction of $MDION^+Cl^-$ in the enzymatic (CPR-mediated) process. However, in the case of $MNP^+Cl^-$ the appearance of the absorption band ($\lambda_{max}$ = 445 nm) characteristic for the chemically reduced form of MNP salt was not observed. This could suggest that the CPR-mediated reduction of $MNP^+Cl^-$ occurs in a different manner in comparison with the non-enzymatic reduction of this salt. This assumption is in agreement with data from our previous report showing that the non-enzymatic reduction of $MNP^+$ in the presence of NADPH via a transhydrogenation reaction involves two-electron transfer resulting in the formation of MNPH, whereas the enzymatic process (xanthine oxidase-mediated) involves one-electron reduction of $MNP^+$ to $MNP^\bullet$ radical, which in turn can be reoxidized back to $MNP^+$ or dimerize to form $(MNP)_2$ (Wieczorkowska et al 2003).

The effect of doxorubicin and vincristine on the non-enzymatic reduction of $MNP^+Cl^-$ and $MDION^+Cl^-$ as well as CPR-dependent reduction of $MDION^+Cl^-$ was studied by the absorption measurements carried out continuously for each sample studied at selected absorption wavelengths, 445 and 410 nm for MNP and MDION salts, respectively, representing the maximum absorption wavelengths of their reduced forms (Figure 4). It was found that vincristine had no effect on non-enzymatic reduction of $MNP^+Cl^-$ and $MDION^+Cl^-$ (data not presented) as well as CPR-mediated reduction of MDION salt (Figure 4), whereas doxorubicin had no influence on non-enzymatic $MNP^+Cl^-$ and $MDION^+Cl^-$ (data not presented) but drastically inhibited (by about 85%) CPR-dependent reduction of MDION salt (Figure 4). Studying the effect of doxorubicin and vincristine on CPR-dependent reduction of MNP salt by the analogous measurements was not possible because of the lack of the absorption spectrum characteristic for the reduced form of this salt obtained in the enzyme-mediated process.

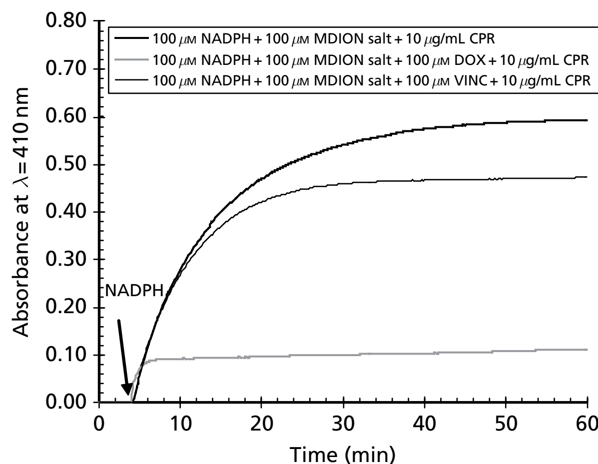


**Figure 4** Spectroscopic changes followed during incubation of 100 μM $MDION^+Cl^-$, with 100 μM doxorubicin (DOX) or 100 μM vincristine (VINC) in enzymatic (CPR-dependent) system. The selected wavelength (410 nm) represents the maximum absorption wavelength for the reduced form of MDION salt. The measurements were carried out in 0.01 M $K_2HPO_4/KH_2PO_4$ buffer (pH 7.25) at 37°C. The reactions were initiated by the addition of 100 μM NADPH. Data shown are from a representative experiment.

## Conclusions

The presented data suggest that the bioreductive drug doxorubicin, in contrast to vincristine, could compete with pyridinium salts ($MNP^+Cl^-$ and $MDION^+Cl^-$) for NADPH-dependent oxidoreductases and for undergoing cellular reductive activation. It could explain the inefficiency of these salts to increase the cytotoxic activity of doxorubicin against the examined leukaemic HL60 cell line and its resistant sublines, HL60/VINC and HL60/DOX. However, to verify this assumption, further studies with the use of various bioreductive and non-bioreductive drugs should be performed. Moreover, to demonstrate the generalizability of the presented findings, the effect of pyridinium salts on the cytotoxic activity of anti-tumour agents against a larger panel of cell lines should be studied and the in-vitro data should be confirmed in in-vivo models.

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