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Communication

The effect of new non-cross resistant antitumour agents on the energy state of human erythrocytes

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Multidrug resistance (MDR) of tumour cells is related to the overexpression of ATP-dependent pumps responsible for the active efflux of antitumour agents out of resistant cells. Benzoperimidine and anthrapyridone compounds exhibit comparable cytotoxic activity against sensitive and MDR tumour cells. They diffuse extremely rapidly across the plasma membrane and render the ATP-dependent efflux inefficient. Such uptake could disturb an energy metabolism of normal cells possessing an elevated level of ATP-dependent proteins, especially erythrocytes having a high level of the MRP1, MRP4 and MRP5 proteins. In this study the effect of five antitumour agents: benzoperimidine (BP1), anthrapyridones (CO1, CO7) and reference drugs used in the clinic: doxorubicin (DOX) and pirarubicin (PIRA), on the energetic state in human erythrocytes has been examined. These compounds have various types of structure and kinetics of cellular uptake (slow – DOX, CO7, moderate – PIRA, fast – BP1, CO1) resulting in their different ability to saturate ATP-dependent transporters. The energetic state of erythrocytes was examined by determination of purine nucleotide contents (ATP, ADP, AMP), NAD^+ and values of adenylate energy charge (AEC) using an HPLC method. It was found that the level of nucleotides as well as the AEC value of erythrocytes were not changed during 24 h of incubation with these agents independently of their structure and ability to saturate ATP-dependent pumps. This is a very promising result in view of their potential use in the clinic as antitumour drugs against multidrug resistant cancers.

Keywords: multidrug resistance, ATP-dependent efflux, non-cross resistant antitumour benzoperimidines and anthrapyridones, erythrocytes, energy state

The appearance of multidrug resistance (MDR) of tumour cells to a wide array of antitumour drugs, structurally diverse and having different mechanisms of action (e.g., anthracyclines, *Vinca* alkaloids, podophylotoxins, taxanes), constitutes the major obstacle to successful treatment of cancer (Chaudhary & Roninson, 1993). For this reason the search for new antitumour agents able to overcome multidrug resistance is of prime importance. The MDR phenotype is associated with the overexpression of ATP-dependent drug efflux pumps (e.g., P-glycoprotein, MRP1, BCRP/MXR1) (Borst *et al.*, 2000; Litman *et al.*, 2001; Ejendal & Hrycyna, 2002; Plasschaert *et al.*, 2003). These transporters are responsible for the active efflux of antitumour drugs out of resistant cells resulting in a significant decrease in their intracellular accumulation, insufficient to inhibit proliferation of resistant cells (Paul *et al.*, 1996).

The cytotoxic activity depends on drug concentration in the compartment where its cellular target is located. The intracellular drug concentration is determined by the kinetics of cellular uptake and the kinetics of ATP-dependent export of the drug (Frezard & Garnier-Suillerot, 1991; Garnier-Suillerot, 1995). For clinically used anthracyclines (doxorubicin, DOX; daunorubicin, DNR; pirarubicin, PIRA), it was found that the rate of passive diffusion was comparable to the rate of P-glycoprotein- and MRP1-mediated efflux leading to a sig-

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Abbreviations: AEC, adenylate energy charge; BP1, 8,11-dihydroxy-[(2-dimetylamino)ethyl]amino-7*H*-benzo[e]perimidin-7-one; CO1, 4-[[2-(dimethylamino)ethyl]amino]-*N*-methyl-1,9-anthrapyridone; CO7, 4-[(2-aminoethyl)amino]-*N*-metyl-1,9-anthrapyridone; DOX, doxorubicin; FBS, fetal bovine serum; PIRA, pirarubicin (4'-*O*-tetrahydropyranyl-doxorubicin).

nificant decrease in drug accumulation in resistant cells (Mankhetkorn *et al*., 1996; Marbeuf-Gueye *et al*., 1998). An increase in drug accumulation in resistant cells could be achieved by an augmentation of the kinetics of its passive uptake into the cells or by a reduction of their affinity to the exporting pumps, resulting in a decrease in the kinetics of active efflux.

Our approach to search for antitumour agents retaining activity against MDR cells concerns the design of derivatives which, due to their physicochemical properties, diffuse very rapidly across the plasma membrane and, in consequence, render the ATP-dependent efflux inefficient. According to this approach, we have recently synthesised new families of benzoperimidines and anthrapyridones. In our previous works (Stefanska *et al*., 1999; Tkaczyk-Gobis *et al*., 2001; Tarasiuk *et al*., 2002) the interaction of these derivatives with several tumour cell lines: erythroleukemia K562 sensitive and K562/DOX resistant (overexpressing P-glycoprotein) as well as small-cell lung cancer GLC4 sensitive and GLC4/DOX resistant (overexpressing MRP1/LRP proteins) cells was studied. It was evidenced that some of these compounds (e.g., benzoperimidine, BP1, anthrapyridone, CO1) exhibited comparable cytotoxic activity against sensitive and resistant tumour cells. We have also confirmed that, for these agents, the kinetics of passive cellular uptake exceeds largely the rate of P-glycoprotein- as well as MRP1-dependent efflux. However, such uptake could disturb the energy equilibrium of tumour as well as normal cells having an increased level of ATP-dependent pumps, especially erythrocytes expressing a high level of the MRP1, MRP4 and MRP5 proteins (Rychlik *et al*., 2000; 2003; Abraham *et al*., 2001; Bobrowska-Hagerstrand *et al*., 2001; Klokouzas *et al*., 2003; Wu *et al*., 2005). This would be a serious problem in view of their potential use as antitumour agents in the treatment of multidrug resistant cancers.

The aim of this study was to examine the effect of selected benzoperimidine (BP1) and anthrapyridone (CO1, CO7) antitumour agents as well as reference drugs used in the clinic (DOX, PIRA) on the energy state of erythrocytes isolated from human peripheral blood. These compounds have various types of structure (Fig. 1) and kinetics of cellular uptake (slow – DOX, CO7, moderate – PIRA, fast – BP1, CO1) resulting in their different ability to saturate ATP-dependent transporters (Marbeuf-Gueye *et al*., 1999; Tkaczyk-Gobis *et al*., 2001; Tarasiuk *et al*., 2002). The energetic state of erythrocytes was examined by determination of purine nucleotide contents (ATP, ADP, AMP), NAD^+ and values of adenylate energy charge (AEC) using an HPLC method. It seems that changes in the level of purine compounds may characterise well the disturbance in energy metabolism in different abnormal cellular states. The same type of studies was largely used by many authors to examine altered cellular metabolism due to, e.g., inherited disorders (Simmonds *et al*., 1998), chronic renal failure (Angle *et al*., 1985) or oxidative stress (Bozzi *et al*., 1994).

MATERIALS AND METHODS

Drugs and chemicals. BP1 was obtained according to Stefanska *et al.* (1999), CO1 and CO7 were prepared according to Stefanska, as described in Tarasiuk *et al.* (2002). The structures and purity of these compounds were confirmed by melting point, IR, ^1H NMR ($CDCl_3$) and MS-FD (mass spectrometry, field desorption technique) analysis. Doxorubicin (DOX) and pirarubicin (PIRA) were kindly provided by Pharmacia-Upjohn. Stock solutions (100 μM) were prepared just before use.

HPLC-grade reagents: acetonitrile (Merck), potassium dihydrogen orthophosphate, dipotassium hydrogen orthophosphate, potassium chloride and tripotassium orthophosphate (Fluka) and purine standards (Sigma-Aldrich) were used. HPLC-grade water was prepared using a Millipore purification system.

Isolation of erythrocytes. Human venous blood samples obtained from healthy adult subject were collected in heparinized tubes and centrifuged at 1500 × *g* for 10 min at 4°C. Plasma with the upper layer containing leukocytes and platelets were decanted. The erythrocyte pellet was washed three times with 3–4 volumes of 0.9% NaCl solution (Ataullakhanov *et al*., 1996). Washed erythrocytes were suspended in 0.9% NaCl to obtain a hematocrit value of 40–50% and counted using Burker hemocytometer. Thereafter erythrocytes were resuspended in RPMI 1640 (Gibco Ltd) medium supplemented with 2 mM glutamine and 10% FBS (Gibco Ltd) to obtain a final concentration of 10^7/ml and incubated in a humidified atmosphere of 95% air and 5% CO_2 at 37°C in the presence of 1 μM examined compound. Samples for counting erythrocyte number (using Burker hemocytometer) and HPLC analysis were taken at indicated times of incubation: 0 (t_0 — addition time of examined agent to the erythrocyte sample), 20 min, 1, 2 and 24 h. For each determination, a sample incubated under the same conditions without any compound was analysed as a control. For some experiments erythrocyte samples were incubated at 37°C with neither RPMI medium nor CO_2 atmosphere.

HPLC separation of nucleotides. Erythrocyte samples (500 μl) were treated with 500 μl of 1.3 M perchloric acid in 1.5 ml Eppendorf tubes. The obtained mixtures were centrifuged at 16000 × *g* for 10 min at 4°C. The supernatant (600 μl) was neutralised with 3 M potassium orthophosphate solution.

Samples were frozen immediately at collection time and unfrozen just before the analysis. Nucleotide concentrations (ATP, ADP, AMP, NAD^+) were determined using an HPLC method (Smolenski *et al.*, 1990). Aliquots of 20 μl of the obtained samples were injected onto the chromatograph column and nucleotides were separated using a linear phosphate buffer gradient elution system (buffer A, 150 mM KH_2PO_4, 30 mM K_2HPO_4, 150 mM KCl, pH 6.0; buffer B, 15% (v/v) solution of acetonitrile in buffer A) at a flow rate of 0.666 ml/min. Peaks were detected by absorbance measurements at 254 nm. The cycle time was 12.8 min between injections. The analytical column was maintained at a constant temperature of 20.5°C. Sample peaks were integrated, calibrated and quantified using an HPLC 2D chromatography data system operating on a Chemstation Software for Windows 98 (Hewlett Packard).

For each sample the adenylate energy charge (AEC) value was calculated according to the following formula (Atkinson, 1968):

$$AEC = \frac{[ATP] + 0.5 \times [ADP]}{[ATP] + [ADP] + [AMP]}$$

Statistical analysis. Results are presented as the mean ± S.D. of five independent experiments. Statistical analysis was performed using Kruskal-Wallis ANOVA test. $P \leq 0.05$ was considered as a significant difference.

RESULTS AND DISCUSSION

Benzoperimidine BP1 and anthrapyridone CO1 retain high activity against multidrug resistant tumour cells. These compounds, due to their physicochemical properties, diffuse extremely quickly into the cell and cause a rapid saturation of ATP-dependent exporting pumps (Tkaczyk-Gobis *et al.*, 2001; Tarasiuk *et al.*, 2002). This could disturb the energy metabolism of cells and be toxic to some normal cells exhibiting an elevated level of ATP-dependent proteins. Erythrocytes express relatively high levels of the MRP1, MRP4 and MRP5 proteins (Rychlik *et al.*, 2000; 2003; Abraham *et al.*, 2001; Bobrowska-Hagerstrand *et al.*, 2001; Klokouzas *et al.*, 2003; Wu *et al.*, 2005). The disturbance in nucleotide metabolism could result in the loss of flexibility, deformation and destruction of erythrocytes (Birchmeier & Singer, 1977). Measurement of erythrocyte nucleotide levels is simple and rapid (Smolenski *et al.*, 1990), allowing one to monitor the consequences of therapy during patient treatment. It seems also very useful to predict the influence of new antitumour compounds on the nucleotide metabolism in view of their possible toxicity limiting clinical application.

Thus, we have examined the effect of five antitumour agents: benzoperimidine (BP1), anthrapyridones (CO1, CO7) and reference drugs used in the clinic, doxorubicin (DOX) and pirarubicin (PIRA), on the level of purine nucleotides in human erythrocytes. These compounds have various types of structure (Fig. 1) and kinetics of cellular uptake, resulting in their different ability to saturate ATP-dependent drug efflux pumps (Marbeuf-Gueye *et al.*, 1999; Tkaczyk-Gobis *et al.*, 2001; Tarasiuk *et al.*, 2002). Benzoperimidine BP1 and anthrapyridone CO1 possess very fast kinetics of cellular uptake exceeding largely the rate of ATP-dependent efflux by cellular pumps (P-glycoprotein, MRP1). In contrast, anthrapyridone CO7, of the same type of structure as CO1, has very slow kinetics of cellular uptake (this derivative was used as a negative control for CO1). Doxorubicin (DOX) has slow kinetics of cellular uptake, and pirarubicin (PIRA) exhibits faster kinetics of cellular uptake than DOX but about 5-times slower in comparison to BP1 and CO1.

Human erythrocytes samples (10^7/ml) isolated from peripheral blood were incubated with 1 μM examined agent. This compound concentration was chosen because, according to literature data, this is a concentration of anthracycline drugs clinically achievable and recommended for *in vitro* studies with intact cells for reflecting the mechanism of

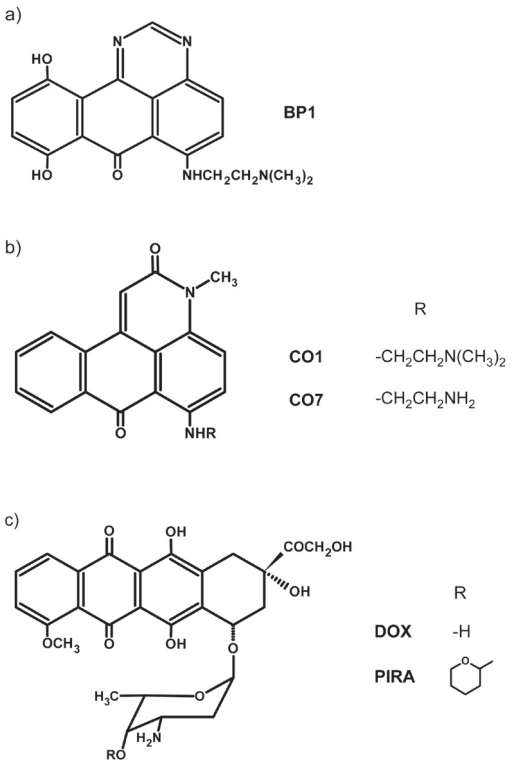

Figure 1. The structures of examined compounds.
a) Benzoperimidine, **BP1** (8,11-dihydroxy-[(2-dimetylamino)ethyl]amino-7*H*-benzo[e]perimidin-7-one); b) anthrapyridones: **CO1** (4-[[2-(dimethylamino)ethyl]amino]-*N*-methyl-1,9-anthrapyridone) and **CO7** (4-[(2-aminoethyl) amino]-*N*-metyl-1,9-anthrapyridone); c) anthracyclines: doxorubicin, **DOX,** and pirarubicin, **PIRA** (4'-O-tetrahydropyranyl-doxorubicin).

Table 1. The content of purine nucleotides (ATP, ADP, AMP) and NAD^+ in human erythrocytes during incubation with examined compounds

Drug	Time of incubation				
	t_0	t_1 = 20 min	t_2 = 1 h	t_3 = 2 h	t_4 = 24 h
ATP [pmol/10^6 erythrocytes]					
–	121.8 ± 10.8	105.7 ± 19.6	104.3 ± 19.3	110.1 ± 18.4	107.5 ± 19.9
BP1	116.1 ± 28.8	113.2 ± 13.5	117.4 ± 14.0	92.4 ± 10.7	109.5 ± 13.1
CO1	112.4 ± 16.1	116.3 ± 10.7	122.2 ± 11.3	140.5 ± 4.4	117.8 ± 10.9
CO7	108.6 ± 8.0	114.9 ± 6.8	121.2 ± 7.2	115.0 ± 4.9	114.5 ± 6.8
DOX	107.7 ± 9.7	101.7 ± 15.5	106.7 ± 16.3	91.1 ± 5.1	102.0 ± 15.6
PIRA	125.4 ± 10.2	111.3 ± 8.3	109.9 ± 8.2	123.7 ± 10.1	119.7 ± 9.0
Positive control*	120.4 ± 0.8	111.7 ± 5.8	70.9 ± 1.0	34.4 ± 2.7	n.d.
ADP [pmol/10^6 erythrocytes]					
–	16.4 ± 2.9	17.0 ± 5.1	20.3 ± 6.1	18.2 ± 3.6	17.9 ± 5.4
BP1	16.0 ± 2.0	17.6 ± 2.1	21.8 ± 2.6	14.9 ± 2.0	17.1 ± 2.0
CO1	12.1 ± 2.9	16.5 ± 1.8	18.7 ± 2.1	23.2 ± 5.9	14.9 ± 1.8
CO7	11.4 ± 0.9	16.0 ± 2.2	20.0 ± 2.8	15.6 ± 0.5	16.4 ± 2.2
DOX	18.3 ± 5.8	19.2 ± 4.8	17.4 ± 4.4	19.4 ± 4.3	19.3 ± 4.9
PIRA	15.0 ± 2.7	15.8 ± 1.9	17.6 ± 2.2	16.1 ± 1.1	18.4 ± 1.9
Positive control*	16.1 ± 1.8	27.4 ± 1.1	50.0 ± 0,3	24.3 ± 2.6	n.d.
AMP [pmol/10^6 erythrocytes]					
–	1.6 ± 0.2	2.0 ± 0.4	2.5 ± 0.5	2.9 ± 0.6	2.1 ± 0.4
BP1	1.8 ± 0.1	2.5 ± 0.4	3.4 ± 0.6	3.3 ± 0.6	2.5 ± 0.5
CO1	1.8 ± 0.2	2.1 ± 0.2	2.9 ± 0.3	2.1 ± 0.3	1.7 ± 0.2
CO7	2.3 ± 0.6	1.9 ± 0.3	2.3 ± 0.4	2.6 ± 0.4	1.9 ± 0.3
DOX	2.0 ± 0.5	2.6 ± 0.7	3.5 ± 0.9	2.1 ± 0.3	2.6 ± 0.7
PIRA	1.5 ± 0.1	2.1 ± 0.3	3.1 ± 0.4	2.1 ± 0.9	2.6 ± 0.3
Positive control*	1.7 ± 0.1	2.5 ± 1.0	7.6 ± 1.4	5.6 ± 0.3	n.d.
NAD^+ [pmol/10^6 erythrocytes]					
–	5.1 ± 1.1	5.2 ± 1.2	6.1 ± 1.5	5.3 ± 1.2	5.4 ± 1.3
BP1	5.1 ± 1.3	4.9 ± 0.6	6.5 ± 0.8	5.3 ± 1.0	5.3 ± 0.7
CO1	5.1 ± 1.3	5.2 ± 0.6	5.7 ± 0.6	5.2 ± 0.6	5.6 ± 0.7
CO7	5.3 ± 2.0	5.2 ± 0.5	6.1 ± 0.7	4.8 ± 0.3	5.7 ± 0.6
DOX	4.4 ± 0.8	4.0 ± 0.9	4.6 ± 1.0	4.6 ± 0.9	4.4 ± 1.0
PIRA	5.3 ± 0.4	5.8 ± 1.9	6.7 ± 2.2	4.9 ± 0.8	5.8 ± 1.9
Positive control*	5.5 ± 0.6	5.7 ± 0.4	6.6 ± 0.1	4.0 ± 0.3	n.d.

The examined compound at 1 μM was added to an erythrocyte sample and incubated in RPMI medium at 37°C in a humidified atmosphere of 95% air and 5% CO_2; *erythrocyte sample incubated at 37°C in isotonic NaCl; the values are the mean ± S.D. of five independent experiments; n.d. – not determined.

drug actions associated with their clinical utilization (reviewed in Gewirtz, 1999). The same concentration of examined compounds (1 μM) was also employed in our previous studies on their interaction with ATP-dependent exporting pumps present in MDR tumour cell lines: K562/DOX and GLC4/DOX (Tkaczyk-Gobis *et al.*, 2001; Tarasiuk *et al.*, 2002). In consequence, the initial quantities of the agents studied (1 nmol/ml) and total ATP present in erythrocyte suspension (1.22 ± 0.11 nmol/ml) were comparable. For each determination a sample of erythrocytes incubated under the same conditions without any compound was also analysed as a control. Since pharmacokinetic studies have revealed that anthracycline drugs are cleared with an elimination half-life of 20–30 h and 15–20 h for DOX and PIRA, respectively (Robert, 1988; Mader *et al.*, 1995; Gabizon *et al.*, 2003), the energy state of erythrocytes was studied for up to 24 h of incubation with the agents. Control assays performed for erythrocyte samples incubated in the absence or in the presence of examined agents showed that 100% of the number of erythrocytes was present at the end of incubation (not shown), despite these compounds exerting a cytotoxic effect against tumour cells at nanomolar (BP1, DOX, PIRA: 4–10 nM for sensitive and 7–670 nM for resistant cells) or micromolar (CO1, CO7: 0.7–1.8 μM for sensitive and 2–62 μM for resistant cells) concentrations (Tkaczyk-Gobis *et al.*, 2001; Tarasiuk *et al.*, 2002).

Results presented in Table 1 show that for all the examined agents the contents of purine nucleotides (ATP, ADP, AMP), NAD^+ as well as the value of AEC did not change during 24 h of incubation ($P > 0.05$). It is also important to note that the levels of purine nucleotides found for erythrocytes incubated under experimental conditions employed in

the study are in agreement with their physiological concentrations (Traut, 1994). These results show that incubation of erythrocytes in RPMI 1640 medium (supplemented with 2 mM glutamine and 10% FBS) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C did not modify the energy metabolism of erythrocytes, at least up to 24 h. In contrast, erythrocyte samples incubated for 2 h at 37°C with neither RPMI medium nor CO_2 atmosphere showed a significant drop in ATP (71.4%) and NAD^+ (27.3%) levels (Table 1). Simultaneously for these samples significant increases in ADP (50.9%) and AMP (229.4%) contents (Table 1) as well as the drop of 32.4% in the AEC value (from 0.929 ± 0.006 at t_0 to 0.628 ± 0.049 at t_{2h}) were observed. These results found for the "positive control" show that the HPLC method used in the study could detect changes in the content of nucleotides and thus accurately identify changes in the energy state of erythrocytes.

CONCLUSION

Our results show that all the examined agents (independently of their structure and ability to saturate ATP-dependent pumps) have no significant influence on the energy state of erythrocytes. The contents of purine nucleotides (ATP, ADP, AMP), NAD^+ as well as the value of adenylate energy charge (AEC) were not changed during 24 h of incubation of erythrocyte samples with the examined agents. This is a very promising result in view of their potential use in the clinic as antitumour drugs against multidrug resistant cancers.

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