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# The role of structural factors in the kinetics of cellular uptake of pyrazoloacridines and pyrazolopyrimidoacridines Implications for overcoming multidrug resistance towards leukaemia K562/DOX cells

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**Abstract**

The appearance of multidrug resistance (MDR) of tumour cells to a wide array of antitumour drugs, structurally diverse and having different mechanisms of action, constitutes the major obstacle to the successful treatment of cancer. Our approach to search for non-cross resistant antitumour agents is based on the rational design of derivatives, which have a high kinetics of passive cellular uptake rendering their active efflux by MDR exporting pumps inefficient. Recently, two families of acridine cytotoxic agents were obtained, pyrazoloacridines (PACs) and pyrazolopyrimidoacridines (PPACs). The aim of this study was to examine molecular basis of the reported differences in retaining cytotoxic activity of these derivatives at cellular level against resistant erythroleukaemia K562/DOX (overexpressing P-glycoprotein) cell line. The study was performed using a spectrofluorometric method, which allows continuous monitoring of the uptake and efflux of fluorescent molecules by living cells. It was demonstrated that the presence of two additional rings, pyrazole and pyrimidine, fused to the acridine chromophore structure (PPAC) favoured more rapid cellular diffusion than the presence of only one additional pyrazole ring (PAC). The presence of hydrophobic subsituent $OCH_3$ markedly favoured the cellular uptake of pyrazoloacridines and pyrazolopyrimidoacridines while compounds having hydrophilic substituent OH exhibited very low kinetics of cellular uptake. In contrast, it was found that neither structure of the ring system nor the hydrophobic/hydrophilic character of examined substituents determined the rate of active efflux of these compounds by P-glycoprotein. Our data showed that a nearly linear relation exists between the resistance factor (RF) and $\ln V_+$ reflecting the impact of the cellular uptake rate ($V_+$) on the ability of these compounds to overcome MDR.


*Keywords:* Multidrug resistance; P-glycoprotein; Non-cross resistant antitumour agents; Acridine cytotoxic agents; Pyrazoloacridines (PACs); Pyrazolopyrimidoacridines (PPACs)

## 1. Introduction

Multidrug resistance (MDR) constitutes the major problem in cancer therapy. Tumour cells become resistant to a wide array of chemotherapeutic agents (e.g. anthracyclines, vinca alkaloids, podophylotoxins, colchicine), structurally diverse and having different mechanisms of action [1]. Multidrug resistance phenomenon is associated with the presence of membrane proteins belonging to the ATP-binding cassette protein family (P-glycoprotein, multidrug resistance-associated protein family MRPs) [2–4]. 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 [5,6].

One of the most investigated strategies to reverse MDR is based on the use of MDR reversing agents (chemosensitizers) able to modulate the function of MDR exporting

*Abbreviations:* DOX, doxorubicin; DR, daunorubicin; MDR, multidrug resistance; MRP1, multidrug resistance protein; PACs, pyrazoloacridines; PPACs, pyrazolopyrimidoacridines; PIRA, pirarubicin; P-gp, P-glycoprotein

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pumps [7]. Although many compounds were shown to be inhibitors of P-glycoprotein activity, only few of them are active against MRP1 [4,8].

Our approach to overcome the cell resistance is the design and synthesis of new non-cross-resistant drugs having physicochemical properties that enhance the uptake of drugs by resistant cells [9,10]. This approach is based on the fact that the intracellular drug concentration attainable in resistant cells depends on the difference between the rate of passive cellular uptake and the rate of active MDR transporter-mediated efflux [11,12]. Thus, it might be possible to design derivatives that for the reason of their physicochemical properties diffuse very rapidly into the cell rendering their active efflux inefficient. We have demonstrated that benzoperimidines [13,14] as well as properly substituted pyrimidoacridones [15] and anthrapyridones [16], exhibiting very high kinetics of cellular uptake, were able to overcome multidrug resistance-mediated either by P-glycoprotein or MRP1. We postulate further that the presence of an additional heterocyclic ring (five-or six-membered) fused to the anthracenedione or acridine ring system is the structural factor, which extremely favours the passive diffusion across the plasma membrane. However, we found that the presence of hydrophobic/hydrophilic substituents can markedly affect the drug transport and the ability to overcome multidrug resistance. In contrast, it seemed that the hydrophobic/hydrophilic character of studied compounds did not determine their affinity for MDR exporting pumps.

In order to verify and generalize our supposition on the importance of additional heterocyclic ring presence and hydrophobic/hydrophilic character of substituents introduced to the chromophore structure of lead compounds in the ability to overcome multidrug resistance, the study of other groups of antitumour compounds having the same type of structure must be evidenced. Recently, two families of acridine cytotoxic agents were obtained: pyrazoloacridines (PACs) and pyrazolopyrimidoacridines (PPACs) [17,18]. PAC derivatives have an additional five-membered pyrazole ring fused to the acridine ring system structure and PPAC compounds contain two additional rings, pyrazole and pyrimidine fused to the acridine chromophore structure. The in vitro cytotoxic activity of the series of properly selected PAC and PPAC derivatives was tested towards a panel of sensitive and resistant human tumour cell lines. It was found that the structure of the ring system and kind of substituents considerably influenced the cytotoxic activity of examined pyrazoloacridines as well as pyrazolopyrimidoacridines against multidrug resistant tumour cells [19].

The aim of this study was therefore to examine the molecular basis of reported differences in retaining cytotoxic activity of pyrazoloacridines and pyrazolopyrimidoacridines at cellular level against anthracycline-resistant erythroleukaemia K562/DOX cells. Our data showed that the presence of additional rings fused to the acridine moiety and hydrophobic/hydrophilic character of substituents introduced to the chromophore structure of PACs and PPACs played the crucial role in their ability to cross biological membranes and thus determined their kinetics of passive cellular uptake into sensitive as well as resistant cells. It was demonstrated that the presence of two additional rings fused to the acridine chromophore structure (PPAC) favoured more rapid cellular diffusion than the presence of only one additional pyrazole ring (PAC). The presence of hydrophobic subsituent $OCH_3$ markedly favoured the cellular uptake of pyrazoloacridines and pyrazolopyrimidoacridines while compounds having hydrophilic substituent OH exhibited very low kinetics of cellular uptake. In contrast, it was found that neither structure of the ring system nor the hydrophobic/hydrophilic character of examined substituents determined the rate of active efflux of these compounds by P-glycoprotein.

## 2. Methods

### 2.1. Drugs and chemicals

Pyrazoloacridines and pyrazolopyrimidoacridones were prepared according to Antonini et al. [17,18]. Pirarubicin was kindly provided by Pharmacia–Upjohn. Stock solutions of compounds in deionised double-distilled water were prepared just before use. Concentrations were determined by diluting stock solutions to approximately 10 μM and using an extinction coefficient values indicated in Table 1. Absorption spectra were recorded on a Beckman spectrophotometer. LysoTracker™ Green DND-26 was purchased from Molecular Probes.

### 2.2. Cell lines and cultures

Anthracycline-sensitive K562 and anthracycline-resistant erythroleukaemia K562/DOX cells [20] were grown in RPMI medium supplemented with L-glutamine and 10% foetal calf serum in a humidified atmosphere of 95% air and 5% $CO_2$. The resistant K562/DOX cells were cultured with 400 nM doxorubicin until one to four weeks before

Table 1
Spectroscopic characteristic of pyrazoloacridines (PACs), pyrazolopyrimidoacridines (PPACs) and reference compound, pirarubicin (PIRA)

| Compound | $\lambda_{ex}$ (nm) | $\lambda_{em}$ (nm) | $\varepsilon$ ($M^{-1}$ $cm^{-1}$) |
|---|---|---|---|
| PAC1 | 350 | 420 | 5750 |
| PAC2 | 345 | 430 | 5600 |
| PAC3 | 345 | 430 | 7500 |
| PPAC1 | 332 | 408 | 11550 |
| PPAC2 | 340 | 420 | 11300 |
| PPAC3 | 338 | 418 | 10500 |
| PIRA | 480 | 590 | 11500 |

$\lambda_{ex}$: extinction wavelength, $\lambda_{em}$: emission wavelength, $\varepsilon$: extinction coefficient.

experiments. Cultures initiated at a density of $10^5$ cells $ml^{-1}$ grew exponentially to about $10^6$ cells $ml^{-1}$ in 72 h. For the experiments, culture was initiated at $5 \times 10^5$ cells $ml^{-1}$ and cells were used 24 h later in order to assure an exponential growth phase. They were counted with a Coulter counter, immediately before use in the assay. Cell viability was assessed by trypan blue exclusion.

NIH/3T3 fibroblasts were obtained from the American Type Culture Collection (Manassas, VA) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum in a humidified atmosphere with 5% $CO_2$ incubator. Before each experiment, cells were plated on 18 mm diameter 0.17 mm thick glass cover-slides, and allowed to grow overnight to reach 75% confluence.

### 2.3. Cellular drug accumulation

The cellular uptake of PACs and PPACs was followed by monitoring the decrease of the fluorescence signal. The characteristic values of extinction and emission wavelengths for these compounds are given in Table 1. This spectrofluorometric method has been largely described previously for anthracycline drugs [21,22] as well as for novel antitumour agents, benzoperimidines and anthrapyridones [14,16]. The incubation of cells with the compound proceeds without compromising cells viability. All experiments were conducted in 1 cm quartz cuvettes containing 2 ml of 20 mM HEPES buffer plus 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose (pH 7.3) at 37 °C. In a typical experiment, $2 \times 10^6$ cells were suspended in 2 ml of HEPES buffer under vigorous stirring. Twenty micro litres of the stock compound solution was quickly added to this suspension yielding a concentration equal to $C_T$ ($C_T$ = 1 μM). The decrease in the fluorescence intensity $F$ at emission wavelength indicated for each derivative in Table 1 was followed as a function of time until the curve $F = f(t)$ reached a plateau and the fluorescence intensity was equal to $F_n$. The initial rate of uptake $V_+$ was determined using the equation $V_+ = (dF/dt)_{t=0} \times C_T/F_0$ where $(dF/dt)_{t=0}$ is the slope of the tangent to the curve $F = f(t)$ and $F_0$ is the fluorescence intensity at $t = 0$. The overall cellular concentration of pyrazoloacridine or pyrazolopyrimidoacridine respectively at the steady state was $C_n = C_T(F_0 - F_n)/F_0$ (see Fig. 2a).

### 2.4. P-glycoprotein-mediated efflux of pyrazoloacridines and pyrazolopyrimidoacridines

Cells ($1 \times 10^6$ $ml^{-1}$) were incubated for 30 min in HEPES buffer in the presence of 10 mM $NaN_3$ and in the absence of glucose and then incubated with examined pyrazoloacridine or pyrazolopyrimidoacridine, respectively ($C_T$ = 1 μM). At the steady state ($F_n$) 5 mM glucose was added yielding a new steady state ($F'_n$). The concentrations of compound were respectively $C_n = C_T(F_0 - F_n)/F_0$ and $C'_n = C_T(F_0 - F'_n)/F_0$.

The rate of P-glycoprotein mediated efflux was determined using the equation $V_{P\text{-}gp} = (dF/dt)_{glu}\ C_T/F_0$ (see Fig. 2b). All fluorescence measurements were made on a Perkin–Elmer LS 5B spectrofluorometer.

### 2.5. Lysosomes labelling

Lysosomes were stained with LysoTracker™ Green DND-26 after incubation with 5 μM pirarubicin (PIRA), PAC2, PPAC1, and PPAC2 (from 1 mM stock solutions in water) for 15 min at 37 °C. Cells were incubated with 100 nM of the probe (from 1 mM stock solution in DMSO) in growth complemented DMEM medium for 30 min at 37 °C and washed in PBS prior to observation. Fluorescence microscopy and imaging was performed on a Zeiss Axiovert 35 inverted microscope. Adherent NIH/3T3 cells were mounted in a perfusion chamber and viewed with a Zeiss Plan–Neofluar 100X oil immersion objective (numerical aperture 1.3). Fluorescence image pairs were detected with a cooled CCD camera (Micromax, Princeton Instruments, Evry, France) with a 12-bit detector (RTEA-1317K, Kodak). LysoTracker™ Green DND-26 and pirarubicin were visualized with standard fluorescein and rhodamin filter sets, respectively. PAC2, PPAC1 and PPAC2 were visualized with standard UV filter set. Image pairs were acquired with an exposure time equal to 1 s. Analysis and displays were performed using IPLab software (Scanalytics, Fairfax, VA).

### 2.6. Statistical analysis

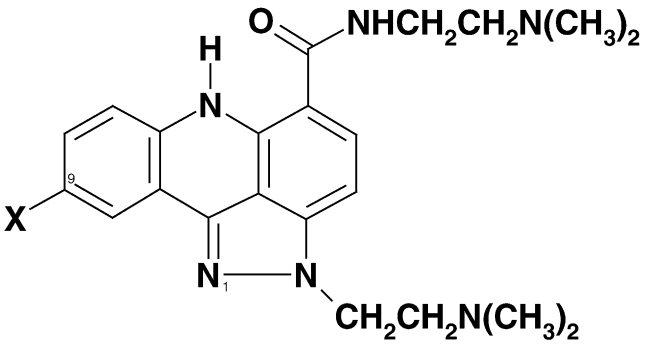
Results are presented as the mean ± S.D. of five independent experiments. Statistical analysis of the significance level of the differences observed between sensitive and resistant cell lines was performed using Mann–Whitney $U$ test. $P < 0.05$ was considered as a significant difference.

## 3. Results

The structures of studied pyrazoloacridines and pyrazolopyrimidoacridines are presented in Fig. 1.

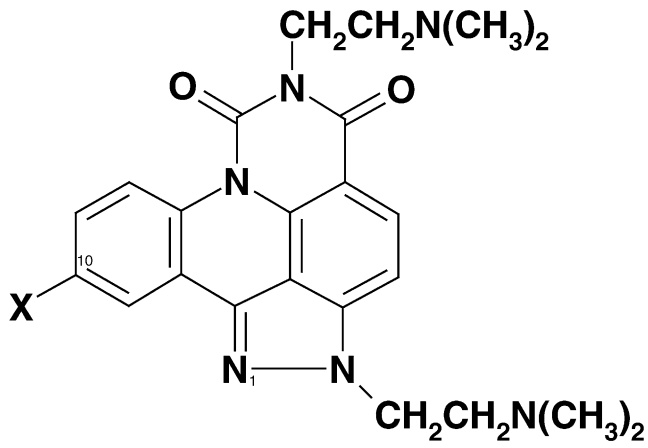
### 3.1. Accumulation of pyrazoloacridines (PACs) and pyrazolopyrimidoacridines (PPACs) in erythroleukaemia K562 sensitive and K562/DOX resistant cells

The accumulation of examined compounds has been studied comparatively in control cells and in energy-depleted cells. Fig. 2a presents the typical fluorescence signals observed when drug-sensitive and drug-resistant K562 cells were incubated in glucose containing HEPES buffer with 1 μM PAC1. During the incubation time a




X = H PAC1
X = $OCH_3$ PAC2
X = OH PAC3

(a)




X = H PPAC1
X = $OCH_3$ PPAC2
X = OH PPAC3

(b)

Fig. 1. Structures of (a) pyrazoloacridone (PAC) and (b) pyrazolopyrimidoacridone (PPAC) derivatives.

significant decrease in the fluorescence signal was observed with K562 sensitive as well as resistant cells due to the cellular uptake of PAC1. The compound concentration bound at the steady state in resistant cells was reduced ($C_n$(R) = 0.50 ± 0.08 μM) in comparison to sensitive cells ($C_n$(S) = 0.74 ± 0.10 μM). At the steady state, 0.02% Triton was added to permeabilize the cellular membranes yielding the identical equilibrium state for sensitive and resistant lines ($C_N$(S,R) = 0.65 ± 0.08 μM).

Fig. 2b shows the record of experiment performed for pyrazoloacridine PAC1 incubated with sensitive and resistant K562 cells in HEPES buffer in the presence of 10 mM $NaN_3$ and in the absence of glucose. Its incorporation at the steady state in these energy-depleted cells was significantly modified in comparison to control cells. The cellular accumulation of compound in energy-depleted resistant cells was almost the same than for energy-depleted sensitive line. After the addition of 5 mM glucose to resistant cells, giving rise to ATP synthesis via the glycolysis pathway, a fast increase of the fluorescence intensity was observed indicating the restoration of P-gp-mediated efflux of the drug. The permeabilisation of cellular membranes with 0.02% Triton gave the identical equilibrium state for sensitive and resistant lines as it was also observed for control cells.

In the same manner, we studied the cellular transport and accumulation for other PACs and PPACs derivatives. All obtained data are reported in Table 2. The $IC_{50}$ for K562 sensitive cell line and resistance factor (RF) values published by Bontemps-Gracz et al. [19] were also added. For comparative purpose, data for reference anthracyclines, daunorubicin and pirarubicin were also presented.

As can be seen, for each compound the initial rate of passive diffusion into sensitive K562 cells was almost the same as for K562/DOX resistant cells but differed markedly depending on the derivative. The 340/360-fold difference was found between the velocity of passive cellular uptake for the derivative penetrating the most rapidly (PPAC2) and the most slowly (PAC3) into the cell suggesting the important role of compound structure in their ability to cross plasma membrane. Comparing appropriate pairs of PAC and PPAC derivatives bearing the same substituents, it appears that pyrazolopyrimidoacridines having two additional rings fused to the acridine chromophore structure penetrated more rapidly into the cell than pyrazoloacridines having only one additional pyrazole ring. It was also found that the presence of hydrophobic/hydrophilic substituents markedly influenced the kinetics of passive cellular uptake of examined PACs as well as PPACs. PAC2 and PPAC2 having the hydrophobic substituent $OCH_3$ penetrated more rapidly into the cell in comparison to their respective non-substituted parent compounds (PAC1 and PPAC1, respectively). In contrast, introduction of the hydrophilic substituent OH to the pyrazoloacridine and pyrazolopyrimidoacridine structures (PAC3, PPAC3) disturbed the passive cellular uptake. For PAC3, the steady state was not reached even after 3 h of incubation with cells while for PPAC2 having the highest rate of cellular uptake the steady state was observed within about 5 min (data not shown).

The rate of P-gp- mediated efflux of these compounds were much less influenced by chemical modifications. The obtained $V_{P\text{-}gp}$ values were very similar for studied derivatives lying in the range (0.49–1.20) × $10^{-18}$ mole/cell/s. Additional studies have been also done with verapamil a well-known P-glycoprotein blocking agent to support the existence of P-gp-mediated efflux of studied pyrazoloacridines and pyrazolopyrimidoacridines. We observed that 10 μM verapamil inhibited the efflux of these compounds to the same extent that for reference compound pirarubicin,

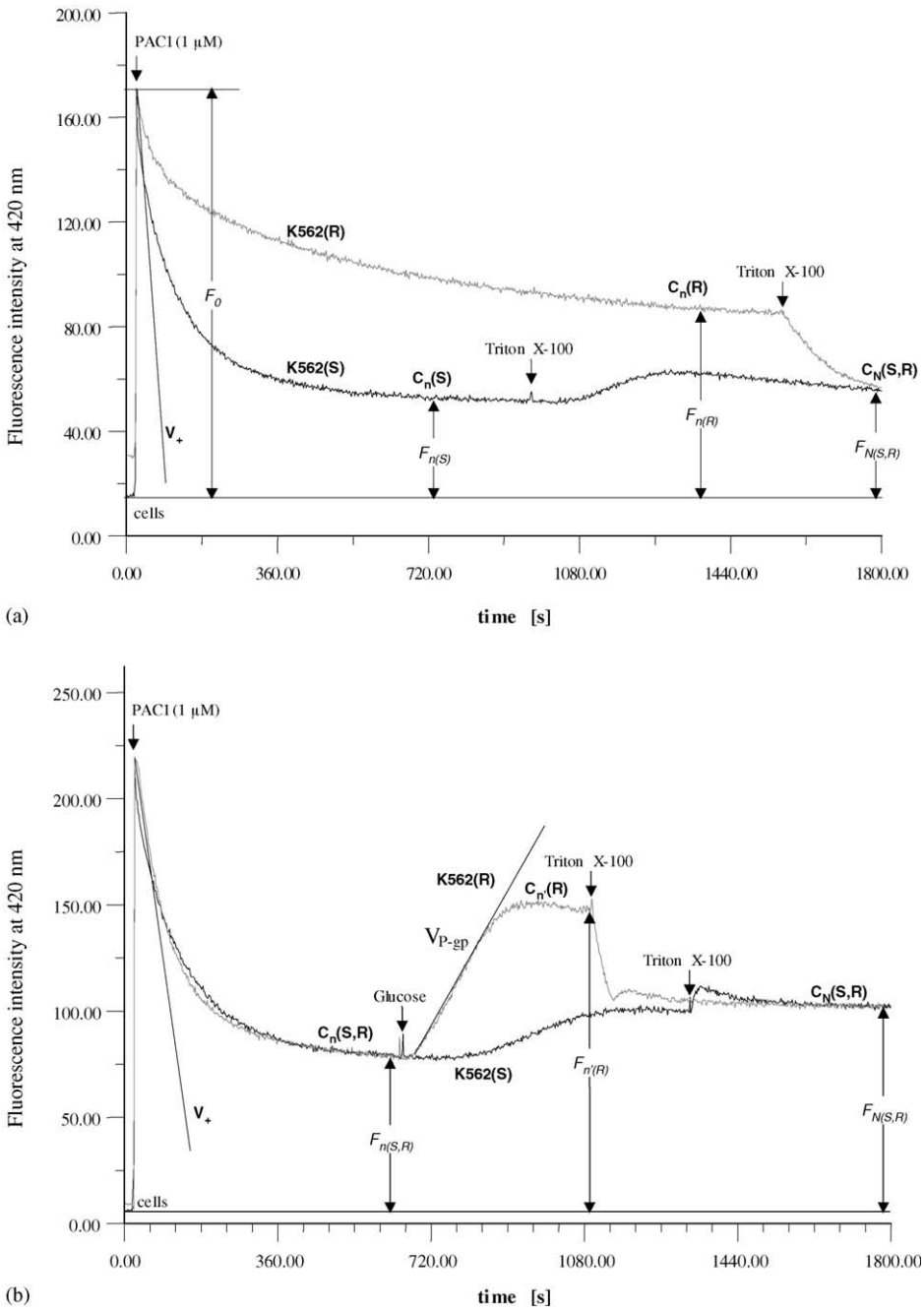



Fig. 2. (a) Uptake of PAC1 by sensitive (S) and resistant (R) K562 cells. Fluorescence intensity at 420 nm ($\lambda_{ex}$ = 350 nm) was recorded as a function of incubation time until the steady state. Cells, $2 \times 10^6$, either sensitive (S) or resistant (R), were suspended in a cuvette-filled with 2 ml buffer at pH 7.25 under vigorous stirring. At $t = 0$, 20 μl of a 100 μM stock PAC1 solution was added to the cell suspension yielding a $C_T = 1$ μM PAC1 solution. At the steady state 0.02% Triton X-100 was added to permeabilize cellular membranes. The experiment was repeated five times and the presented figure is a representative example. (b) Uptake of PAC1 by energy-deprived sensitive (S) and resistant (R) K562 cells. Fluorescence intensity at 420 nm ($\lambda_{ex}$ = 350 nm) was recorded as a function of incubation time. Cells, $2 \times 10^6$, either sensitive (S) or resistant (R), were suspended in a cuvette-filled with 2 ml buffer at pH 7.25 under vigorous stirring and preincubated for 30 min with 10 mM $NaN_3$ in the absence of glucose. At $t = 0$, 20 μl of a 100 μM stock PAC1 solution was added to the cell suspension yielding a $C_T = 1$ μM PAC1 solution. When the steady state was reached, 5 mM glucose was added yielding a new steady state. At the new steady state 0.02% Triton X-100 was added to permeabilize cellular membranes. The rate of P-gp dependent efflux was determined from the slope of the tangent to the curve after the addition of glucose to the resistant cell suspension. The experiment was repeated five times and the presented figure is a representative example.

75% (data not presented). The rate of P-gp-dependent active efflux was not determined for PAC3 and PPAC3 because the steady state for these derivatives was not attainable within 3 h incubation and the prolongation of the incubation time in the presence of 10 mM $NaN_3$ caused the death of cells.

The data presented in Table 2 illustrate very well that the fast passive cellular uptake of PPAC1 and PPAC2 ensured

Table 2
Values of $V_+$(S), $V_+$(R), $V_{P\text{-gp}}$, $C_n$(S), $C_n$(R), $IC_{50}$(S) and RF values for pyrazoloacridones (PACs), pyrazolopyrimidoacridones (PPACs) and reference compounds, daunorubicin and pirarubicin

| Compound | $V_+$(S) ($10^{-18}$ mole/cell/s) | $V_+$(R) ($10^{-18}$ mole/cell/s) | $V_{P\text{-gp}}$ ($10^{-18}$ mole/cell/s) | $C_n$(S) (μM) | $C_n$(R)(μM) | $IC_{50}$(S)[a] (nM) | RF[a] |
|---|---|---|---|---|---|---|---|
| PAC1 | 4.9 ± 1.1 | 5.3 ± 0.6 | 1.2 ± 0.3 | 0.74 ± 0.10 | 0.50 ± 0.08 | 863 ± 70 | 5.1 |
| PAC2 | 5.6 ± 0.7 | 6.8 ± 1.6 | 1.1 ± 0.2 | 0.71 ± 0.03 | 0.52 ± 0.11 | 1192 ± 127 | 2.9 |
| PAC3 | 0.07 ± 0.01 | 0.07 ± 0.01 | n.d. | 0.23 ± 0.02 | 0.13 ± 0.02* | 1012 ± 136 | 16.2 |
| PPAC1 | 15.1 ± 4.2 | 13.3 ± 2.3 | 0.90 ± 0.25 | 0.59 ± 0.07 | 0.52 ± 0.03 | 74 ± 13 | 3.6 |
| PPAC2 | 25.0 ± 6.0 | 23.8 ± 5.9 | 0.49 ± 0.26 | 0.79 ± 0.01 | 0.77 ± 0.08 | 214 ± 37 | 1.7 |
| PPAC3 | 0.32 ± 0.08 | 0.33 ± 0.08 | n.d. | 0.38 ± 0.03 | 0.19 ± 0.02* | 120 ± 6 | 32.1 |
| Daunorubicin | 0.18 ± 0.02 | 0.18 ± 0.02 | 0.15 ± 0.02 | 0.50 ± 0.05 | 0.10 ± 0.02* | 12 ± 2 | 18 |
| Pirarubicin | 3.8 ± 0.3 | 3.8 ± 0.4 | 1.4 ± 0.2 | 0.52 ± 0.05 | 0.29 ± 0.03* | 6 ± 1 | 8 |

$V_+$(S), $V_+$(R) : rate of passive uptake into sensitive (S) and resistant (R) K562 cells; $V_{P\text{-gp}}$: rate of P-gp-mediated efflux of drug; $C_n$(S), $C_n$(R): intracellular drug accumulation in sensitive (S) and resistant (R) cells (at the steady state for PAC1, PAC2, PPAC1 and PPAC2 or after 3 h of incubation for PAC3 and PPAC3); $IC_{50}$(S): compound concentration required to inhibit 50% sensitive cell growth; RF: resistance factor. The values are mean ± S.D. of five independent experiments; n.d.: not determined. The significance level of the differences observed (Mann–Whitney $U$ test): $^*P < 0.05$ versus values found for sensitive cells.
[a] Data published by Bontemps-Gracz et al. [19].

their high accumulation in resistant cells, comparable to that observed in sensitive line. These compounds conserved also a high cytotoxic activity against resistant K562/DOX cells. At the opposite, about two-fold decrease in cellular accumulation was observed in resistant cells in regard to sensitive counterpart for PAC3 and PPAC3 derivatives having the slow kinetics of cellular uptake. The in vitro data showed that they were much less active against resistant K562/DOX cells in comparison to sensitive line. In Fig. 3 the resistance factor values for PAC and PPAC compounds have been plotted as a function of $\ln V_+$. As can be seen, a nearly linear relation exists between RF and $\ln V_+$ reflecting the impact of the cellular uptake rate ($V_+$) on the ability of drugs to overcome MDR.

### 3.2. Intracellular distribution of pyrazoloacridines (PACs) and pyrazolopyrimidoacridines (PPACs) in NIH/3T3 cells

In order to define the intracellular distribution of pyrazoloacridines and pyrazolopyrimidoacridines, NIH/3T3 cells were used, because they have relatively small nucleus and thus the intracellular organelles are easy visualised. Our previous study on intracellular distribution given for benzoperimidines [14] and anthrapyridones [16] showed that these compounds accumulated in the nucleus and in part in lysosomes. Because of the similar physicochemical properties of pyrazoloacridines and pyrazolopyrimidoacridines with that observed earlier for benzoperimidines [14]

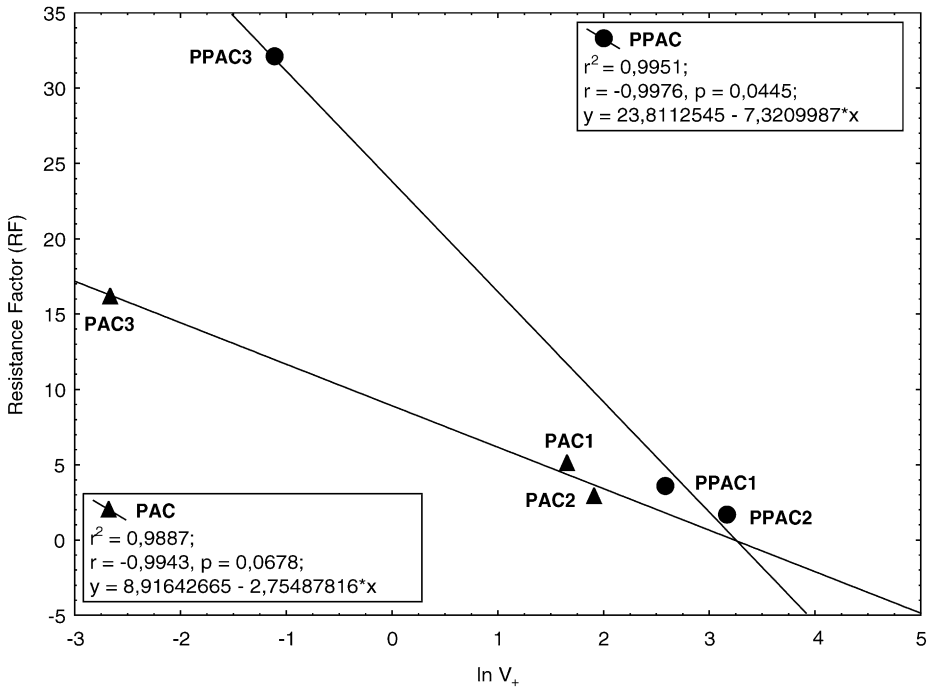



Fig. 3. Relation between the resistance factor (RF) and the rate of cellular uptake $V_+$ for PAC and PPAC compounds. The values of RF have been plotted as a function of $\ln V_+$.

and anthrapyridones [16], we admitted that they would have the similar intracellular distribution. In order to check this assumption cells were incubated with 5 μM compound and then with LysoTracker™ Green DND-26 to stain lysosomes. The series of images obtained by confocal microscopy (Fig. 4) showed the accumulation in the nucleus, as well as, punctuated zones of strong fluorescence signal have been detected. Confocal microscopy analysis using LysoTracker™ Green DND-26, the specific probe for lysosomes, confirmed that these punctuated zones corresponded to the lysosomal accumulation. The same type of imaging analysis was not possible to perform using leukaemia K562 cells. However, additional studies done comparatively with control K562 cells and with K562

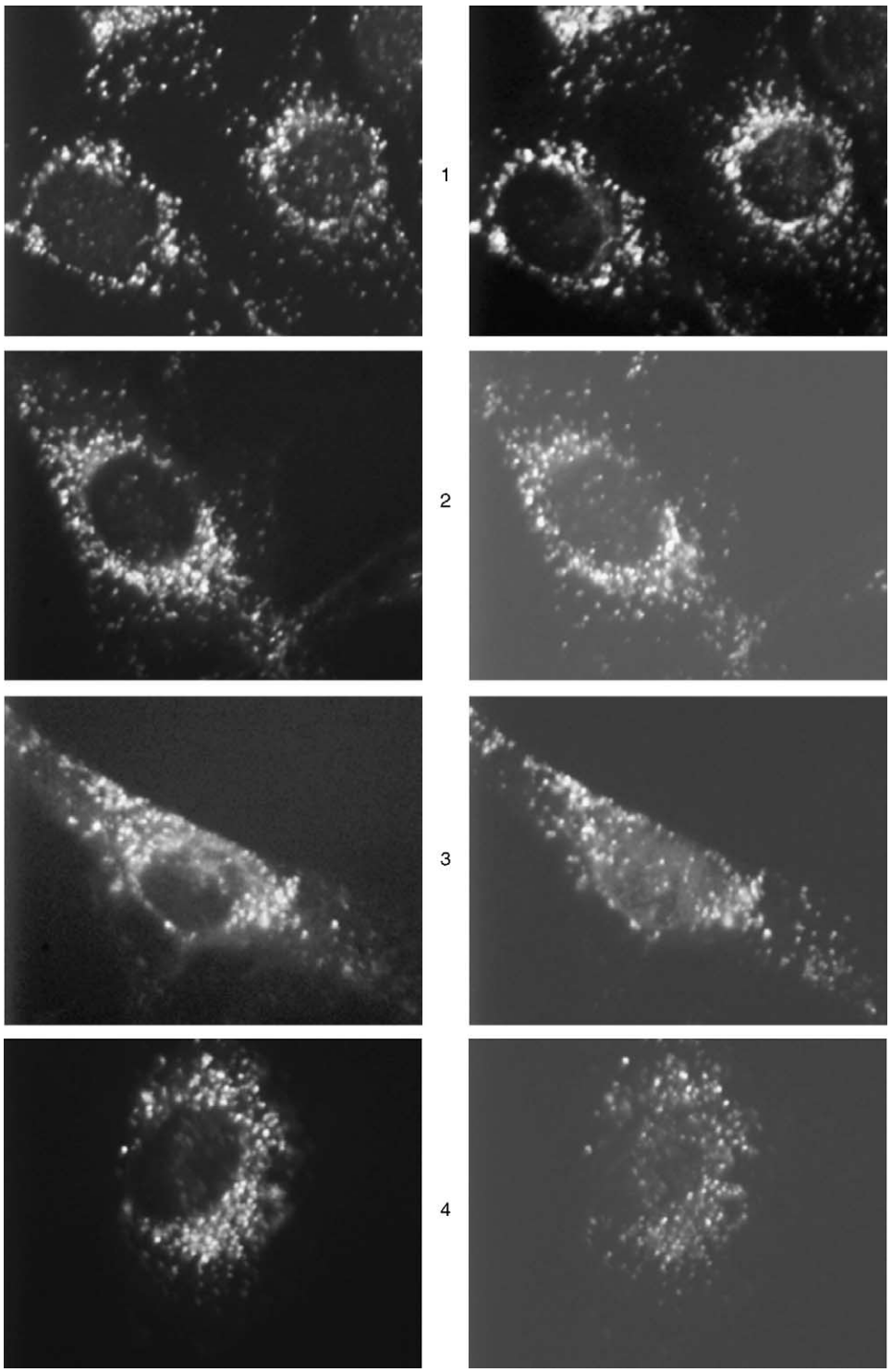



Fig. 4. Co-localization of reference compound, pirarubicin (1), PPAC1 (2), PPAC2 (3) and PAC 2 (4) with lysosomes probe in NIH/3T3 cells. Cells were labelling according to the procedure described in Section 2 with 5 μM compound (right) and specific lysosome probe: LysoTracker™ Green DND-26 (left).

cells in the presence of concanamycin (dissipating the energy-dependent pH gradient of lysosomes) confirmed that besides the nuclear binding of PACs and PPACs, lysosomes are the second important site of their cellular accumulation also in the case of K562 cells (data not presented). The lysosomal accumulation of pyrazoloacridines and pyrazolopyrimidoacridines being weak bases is related to the more acidic pH of the lysosomal compartments (pH $\approx$ 5) than that of the cytoplasm (pH $\approx$ 7.25).

## 4. Discussion

The strategy focused on the search for new non-cross resistant drugs able to overcome MDR is of prime importance. The approach to design antitumour agents not recognized by MDR exporting pumps and in consequence able to the total circumvention of multidrug resistance seems very difficult to realise for the reason that several transporters have been identified in MDR tumour cells [2–4]. Our approach to search for non-cross resistant antitumour agents is based on the design of derivatives which, for the reason of their physiochemical properties, diffuse to the tumour cells with velocity surpassing effective drug efflux by MDR transporters [9–14]. It renders the active efflux inefficient and allows the retaining of the therapeutic concentration of the drug in resistant cells. In consequence the RF is tending to 1. However, it should be mentioned, that the RF close to 1 does not indicate that the drugs are not transported by MDR exporting proteins.

Thus, the identification of structural factors favouring the cellular uptake of antitumour agents is indispensable for the rational design of non-cross resistant drugs. In our previous studies we postulated that the presence of an additional heterocyclic ring (five-or six-membered), fused to the anthracenedione or acridine ring system, was the structural factor that extremely favoured the passive diffusion accross the plasma membrane. However, we found that the presence of hydrophobic/hydrophilic substituents can markedly affect the drug transport and the ability to overcome multidrug resistance [13–16].

In order to further support this hypothesis, two novel families of acridine cytotoxic agents were obtained, pyrazoloacridines and pyrazolopyrimidoacridines [17,18]. PAC derivatives have an additional five-membered pyrazole ring fused to the acridine ring system structure and PPAC compounds contain two additional rings, pyrazole and pyrimidine, fused to the acridine chromophore structure. In the previous work, the in vitro cytotoxic studies given for the panel of MDR cell lines showed that the resistance factor RF values found for examined PACs and PPACs were much lower (0.4/32.1) than for doxorubicin (32.4/196.4). It was also found that the kind of substituents considerably influenced the cytotoxic activity of examined derivatives against multidrug resistant tumour cells. It was evidenced that the hydrophobic substituent $OCH_3$ was optimal for the low resistance factor values for all types of examined MDR tumour cells while the introduction of hydrophilic substituent OH led to decreased cytotoxicity towards MDR cells overexpressing P-glycoprotein, among them against K562/DOX [19]. The aim of this study was to examine molecular basis of the reported differences in retaining cytotoxic activity of PACs and PPACs at cellular level against anthracycline-resistant erythroleukaemia K562/DOX cells.

The data presented in this study show that:

i. The presence of two additional rings fused to the acridine chromophore structure (PPAC) favoured more rapid cellular diffusion than the presence of only one additional pyrazole ring (PAC); the presence of hydrophobic subsituent $OCH_3$ markedly favoured the cellular uptake of examined compounds while derivatives having hydrophilic substituent OH exhibited very low kinetics of cellular uptake.
ii. The rate of P-gp-dependent active efflux of pyrazoloacridines and pyrazolopyrimidoacridines $V_{P\text{-}gp}$ is much less susceptible to modulate by chemical modifications.
iii. A nearly linear relation exists between the resistance factor and $\ln V_+$ reflecting the impact of cellular uptake rate $V_+$ on the ability of these compounds to overcome MDR.
iv. The cellular accumulation of pyrazoloacridines and pyrazolopyrimidoacridines occurs in part in the nucleus, probably through intercalation of the flat condensed rings between the bases pairs; these compounds being weak bases accumulate also in lysosomes.

Results presented in this study were obtained for leukaemia K562/DOX cells overexpressing P-glycoprotein. However data published by Bontemps-Gracz et al. [19] showed that PAC and PPAC derivatives retaining the high activity against K562/DOX (PAC2: RF = 2.9 and PPAC2: RF = 1.7) exhibited also the high activity against promyelocytic leukaemia HL60/DOX overexpressing MRP1 (PAC2: RF = 0.9 and PPAC2: RF = 0.9) while compounds exhibiting diminished activity against K562/DOX (PAC3: RF = 16.2 and PPAC3: RF = 32.1) were also less active against HL60/DOX cells (PAC3: RF = 4.4 and PPAC3: RF = 15.0).

In conclusion: our results obtained for two novel families of antitumour agents, pyrazoloacridines and pyrazolopyrimidoacridines, confirm our assumption that it is possible to design non-cross resistant drugs simply by the fact that they have a very high kinetics of passive cellular uptake rendering the efflux by MDR transporters inefficient. The presented data corroborate also our postulate that the presence of additional heterocyclic rings fused to the acridine ring system and introduction of hydrophobic substituents are structural factors extremely favouring the

passive diffusion of obtained derivatives into the cell. However it should be notified that the present findings can only apply to rapidly proliferating cells and could not be extrapolated on quiescent cells abundant in human tumors.

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