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# Anthrapyridones, a novel group of antitumour non-cross resistant anthraquinone analogues. Synthesis and molecular basis of the cytotoxic activity towards K562/DOX cells

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**1** Multidrug resistance (MDR) to antitumour agents, structurally dissimilar and having different intracellular targets, is the major problem in cancer therapy. MDR phenomenon is associated with the presence of membrane proteins which belong to the ATP-binding cassette family transporters responsible for the active drug efflux leading to the decreased intracellular accumulation.

**2** The search of new compounds able to overcome MDR is of prime importance.

**3** Recently we have synthesized a new family of anthrapyridone compounds. The series contained derivatives modified with appropriate hydrophobic or hydrophylic substituents at the side chain.

**4** The interaction of these derivatives with erythroleukemia K562 sensitive and K562/DOX resistant (overexpressing P-glycoprotein) cell lines has been examined. The study was performed using a spectrofluorometric method which allows to continuously follow the uptake and efflux of fluorescent molecules by living cells.

**5** It was demonstrated that the increase in the lipophilicity of anthrapyridones favoured the very fast cellular uptake exceeding the rate of P-gp dependent efflux out of the cell. For these derivatives, very high accumulation (the same for sensitive and resistant cells) was observed and the *in vitro* biological data confirmed that these compounds exhibited comparable cytotoxic activity towards sensitive and P-gp resistant cell line. In contrast, anthrapyridones modified with hydrophylic substituents exhibited relatively low kinetics of cellular uptake.

**6** For these derivatives decreased accumulation in resistant cells was observed and the *in vitro* biological data demonstrated that they were much less active against P-gp resistant cells in comparison to sensitive cells.

**7** We also studied, using confocal microscopy, the intracellular distribution of anthrapyridones in NIH-3T3 cells. Our data showed that these compounds were strongly accumulated in the nucleus and lysosomes.

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**Keywords:** Multidrug resistance; active efflux; P-glycoprotein; non-cross resistant agents; anthrapyridones; lipophilicity

**Abbreviations:** CO, anthrapyridone; DOX, doxorubicin; MDR, multidrug resistance; MRP1, multidrug resistance protein; PIRA, pirarubicin; P-gp, P-glycoprotein

## Introduction

The ability of neoplastic cells to develop multi-drug resistance (MDR) to chemotherapeutic agents (e.g. anthracyclines, vinca alkaloids, podophylotoxins, colchicine), structurally dissimilar and having different intracellular targets, constitutes the major problem in cancer therapy (Chaudhary & Roninson, 1993). MDR is a well-characterized phenomenon (Beck, 1987; Gottesman & Pastan, 1993), associated with the presence of membrane proteins belonging to the ATP-binding cassette protein family (P-glycoprotein, MRP1, c-MOAT proteins) (Roninson, 1992; Germann, 1996; Loe *et al*., 1996; Kool *et al*., 1997). 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 (Zaman *et al*., 1994; Paul *et al*., 1996).

Current strategies to reverse MDR are based: (i) on the synthesis of new non-cross resistant cytostatics (Antonini *et al*., 1995; Bassan *et al*., 1997; Kubota *et al*., 1998; Stefańska *et al*., 1999); (ii) on the identification of effective and more selective MDR reversing agents (chemosensitizers) (Raderer & Scheithaner, 1993; Pereira *et al*., 1994; Mankhetkorn & Garnier-Suillerot, 1996; Pascaud *et al*., 1998; Berger *et al*., 1999; Teodori *et al*., 1999); (iii) on the selective inhibition of gene expression encoding ATP-dependent export pump using antisense oligonucleotides (Alahari *et al*., 1996; Stewart *et al*.,

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1996; Garcia-Chaumont *et al*., 2000) or ribozymes (Palfner *et al*., 1995).

Because of the continuous identification of new proteins involved in multidrug resistance (Doyle *et al*., 1995; Scheffer *et al*., 1995; Gaj & Yung-Chi, 1998) the approaches (ii) and (iii) seem very far from being resolved. For this reason the search for new compounds able to overcome MDR is of prime importance. 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 drug export of the drug (Frezard & Garnier-Suillerot, 1991; Garnier-Suillerot, 1995). For anthracyclines, it was found that the rate of passive diffusion was comparable to the rate of P-gp and MRP1 mediated efflux leading to the significant decrease in drug accumulation in resistant cells (Marbeuf-Gueye *et al*., 1998). The increase in drug accumulation in resistant cells could be achieved by an augmentation of kinetics of its passive uptake into the cells or by the reduction of their affinity to exporting pumps resulting in the decrease of kinetics of active efflux. It seems that the rational design of compounds having molecular properties which allow the fast kinetics of passive diffusion across the membrane is easier to realise (Mankhetkorn *et al*., 1996; Marbeuf-Gueye *et al*., 1999; Tkaczyk-Gobis *et al*., 2001).

Recently we have synthesized a new family of anthrapyridone compounds. The series of obtained derivatives contained six anthrapyridones modified at the side chain with appropriate hydrophobic or hydrophylic substituents. The interaction of these derivatives with erythroleukemia K562 sensitive and K562/DOX resistant cell lines (over-expressing P-gp) has been examined in this study. Our data showed that the increase in the lipophilicity of anthrapyridone derivatives favoured their fast passive cellular uptake exceeding the rate of P-gp dependent efflux out of the cell. For these derivatives, very high accumulation (the same for sensitive and resistant cells) were observed and the *in vitro* biological data confirmed that these compounds exhibited comparable cytotoxicity towards sensitive and P-gp resistant cell lines. In contrast, anthrapyridones modified with hydrophylic substituents exhibited relatively low kinetics of cellular uptake. For these derivatives decreased accumulation in resistant cells was observed and the *in vitro* biological data showed that they were much less active against P-gp resistant cells in comparison to sensitive cells. In addition, we studied the intracellular distribution of anthrapyridones in NIH-3T3 cells using confocal microscopy. It was found that these compounds were strongly accumulated in the nucleus and lysosomes.

# Methods

## *General procedure for the synthesis of anthrapyridone compounds CO-1, CO-3 – CO-7*

*4-[[2-(dimethylamino)ethyl]amino]-N-methyl-1,9-anthrapyridone (CO-1)* A sample of 0.38 g (1 mmol) of 4-bromo-N-methyl-1,9-anthrapyridone was dissolved in a mixture of 2 ml of 2-(dimethylamino)ethyl amine and was stirred in 80°C under nitrogen atmosphere for 0.5 h. The progress of the reaction was followed by TLC (silica gel) in the solvent system $CHCl_3$/MeOH (5 : 1). The reaction mixture was diluted with $CHCl_3$ and washed with diluted HCl followed by water to remove the excess of amine. The organic layer was dried over $Na_2SO_4$ and evaporated under reduced pressure. The residue was chromatographed on a silica gel column using $CHCl_3$/MeOH (5 : 1) as eluent followed by $CHCl_3$/MeOH/25% aqueous $NH_4OH$ (5 : 1 : 1).

The CO-1 pure product was converted into its hydrochloride salt using an etheral solution of hydrogen chloride, which was then crystallized from MeOH to give CO-1 as a dark red powder (240 mg, 60%): mp 285-7°C dec. IR (KBr, major peaks $cm^{-1}$): 1470, 1510, 1620. $^1$H NMR (as free base, $CDCl_3$): $\delta$ 2.4 (s, 6H); 2.75 (t, 2H, J = 6.5 Hz); 3.55 (q, 2H, I = 6.5 Hz); 3.85 (s,3H); 7.22 (d, 1H, I = 9); 7.4 (s, 1H); 7.68 (d, 1H, I = 9); 7.72 – 7.78 (m, 2H); 8.22 (dd, 1H); 8.5 (dd, 1H); 10.5 (t, 1H). MS-FD m/z (relative intensity, per cent) 348 ($[M+1]^+$, 100) ($[M]^+$, 30). Anal. ($C_{21}H_{21}N_3O_2 \times HCl$) C,H,N. Compounds CO-3 – CO-7 were obtained in the same way using respective aminoalkylamine $RNH_2$ (for R, see Table 1).

Melting points were determinated with a Boeticus PHMK05 apparatus and were uncorrected. Analyses were within 0.4% of the theoretical values and were carried out on a Carlo Erba CHNS-O-EA1108 instrument. IR spectra were recorded on a Brucker IFS66 spectrometer in KBr pellets: $^1$H NMR spectra were taken on a Varian 300 MHz spectrometer using tetramethylsilane as an internal standard. Molecular weights were determined by mass spectrometry (field desorption technique, MS-FD) on a Varian MAT 711 instrument. Column chromatography was performed on silica gel Merck (70 – 230 mesh).

Stock solutions of anthrapyridones in deionized double-distilled water were prepared just before use. Concentrations were determined by diluting stock solutions to approximately 10 $\mu$M and using an extinction coefficient of $\varepsilon_{495} = 6013\ M^{-1}\ cm^{-1}$. Absorption spectra were recorded on a Beckman spectrophotometer.

**Table 1** Synthesis and physicochemical properties of 4-[(aminoalkyl)amino]-N-methyl-1,9 anthrapyridones[(a)]

| *Compound* | *R* | *Reaction conditions* *Time* (hrs) | *temp* (°C) | *Yield* (%) | *Mp* (°C dec.) | *Molecular formula* |
|---|---|---|---|---|---|---|
| CO-1 | $(CH_2)_2N(CH_3)_2$ | 0,5 | 80 | 60 | 285-7 | $C_{21}H_{21}N_3O_2xHC1$ |
| CO-3 | $(CH_2)_3N(CH_3)_2$ | 1 | 80 | 55 | 276-8 | $C_{22}H_{23}N_3O_2xHCIx0{,}5H_2O$ |
| CO-4 | $(CH_2)_2NHCH_2CH_3$ | 1 | 60 | 45 | 270-3 | $C_{21}H_{21}N_3O_2xHCI$ |
| CO-5 | $(CH_2)_2N(CH_2CH_3)_2$ | 1 | 80 | 50 | 283-5 | $C_{23}H_{25}N_3O_2xHC1$ |
| CO-6 | $(CH_2)_2NH(CH_2CH_2OH)$ | 1 | 60 | 35 | 278-80 | $C_{21}H_{21}N_3O_3xHC1x0{,}5H_2O$ |
| CO-7 | $(CH_2)_2NH_2$ | 1 | 60 | 45 | 280-2 | $C_{19}H_{17}N_3O_2xHC1$ |

[(a)] The structures of examined compounds were confined by their spectral data ($^1$H NMR, MS-FD) and by elemental analysis (C,H,N).

### DNA preparation

High molecular weight calf thymus DNA (from Sigma) 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 of $\varepsilon_{260} = 13200\ M^{-1}\ cm^{-1}$ (b.p.).

### Cell lines and cultures

Anthracycline-sensitive K562 and anthracycline-resistant erythroleukemia K562/DOX cells (Tsuruo *et al*., 1986; Mankhetkorn *et al*., 1996) 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 1–4 weeks before experiments. Cultures initiated at a density of $10^5$ cells $ml^{-1}$ grew exponentially to about $10^6$ cells $ml^{-1}$ in 3 days. 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 cells were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.). They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum in a humidified incubator with 5% $CO_2$. Before each experiment, cells were plated on 18 mm diameter glass coverslips and allowed to grow overnight to reach 75% confluence. The cytotoxicity of the compounds was determined by incubating cells ($3 \times 10^5$) with six different concentrations of the compound for 72 hours in standard 48-well plates. The $IC_{50}$ value defined as the concentration which reduced by 50% the growth rate attained by control culture. The resistance factor (RF) was defined as the $IC_{50}$ for the resistant cells divided by the $IC_{50}$ for the sensitive cells.

### Cellular drug accumulation

The cellular uptake of anthrapyridones was followed by monitoring the decrease of the fluorescence signal at 562 nm (566 nm) ($\lambda ex = 495$ nm). This spectrofluorometric method has been largely described previously (Tarasiuk *et al*., 1989; Frezard & Garnier-Suillerot, 1991; Mankhetkorn *et al*., 1996; Marbeuf-Gueye *et al*., 1998). The incubation of the cells with the compound proceeds without compromising cell viability. All experiments were conducted in 1 cm quartz cuvettes containing 2 ml of 20 mM HEPES buffer plus (mM): NaCl 132, KCl 3.5, $CaCl_2$ 1, $MgCl_2$ 0.5, glucose 5 (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. 20 $\mu$l of the stock anthrapyridone solution was quickly added to this suspension yielding an anthrapyridone concentration equal to $C_T$ ($C_T = 1\ \mu M$). The decrease of the fluorescence intensity F at 562 nm (or 566 nm) was followed as a function of time until the curve $F = f(t)$ reached a plateau and the fluorescence intensity was equal to Fn. The initial rate of uptake $V_+$ was determined using the equation $V_+ = (dF/dt)_{t=0} \cdot C_T/F_0$ 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 anthrapyridone at the steady state was $C_n = C_T(F_0 - F_n)/F_0$ (see Figure 2).

### Determination of the rates of energy-dependent processes (non-nuclear accumulation and P-glycoprotein-mediated efflux)

Cells ($1 \times 10^6\ ml^{-1}$) are 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 anthrapyridone ($C_T = 1\ \mu M$). At the steady state ($F_n$) 5 mM glucose was added yielding a new steady state ($F'_n$). The concentration of anthrapyridone were respectively $C_n = C_T(F_0 - F_n)/F_0$ and $Cn = C_T(F_0 - F'_n)/F_0$.

The global rate of energy-dependent processes was determined using the equation $V_E = (dF/dt)_{glu} \cdot C_T/F_0$. The rate of energy-dependent non-nuclear accumulation was determined as $V_E$ for sensitive cells [$V_{E(S)}$] and P-glycoprotein-mediated efflux was calculated as $V_{P\text{-}gp} = V_{E(S)} - V_{E(R)}$ for anthrapyridones CO-1, CO-3, CO-5 (Figure 4) and as $V_{P\text{-}gp} = V_{E(S)} + V_{E(R)}$ for anthrapyridones CO-4, CO-6, CO-7 (Figure 5).

All fluorescence measurements were made on a Perkin-Elmer LS 5B spectrofluorometer.

### Lysosomes and trans-Golgi labelling

Lysosomes were stained with LysoTracker™ Green DND-26 (from Molecular Probes) after incubation with 1 $\mu$M CO-1 for 5 min at 37°C. Cells were incubated with 50 nM of the probe (from 1 mM stock solution in dimethyl sulphoxide (DMSO) in complemented DMEM medium for 30 min at 37°C and washed with PBS prior to observation. The trans-Golgi were stained according to Panago *et al*. (1989). 50 nmol of $C_6$-NBD-ceramide (6-((N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino) caproyl) sphingosine from Molecular Probes) was dissolved in 200 ml of ethanol and injected into 10 ml of 10 mM HEPES-buffered minimal essential medium containing 0.34 mg of defatted bovine serum albumin. The solution was dialysed overnight at 4°C against HEPES-buffered minimal essential medium and aliquoted. After incubation with 1 $\mu$M CO-1, cells were incubated with $C_6$-NBD-ceramide-bovine serum albumin complex for 5 min at 37°C and washed with PBS prior to observation.

Fluorescence microscopy was carried out on a Nikon Optiphot-2 epifluorescence microscope equipped with a Nipkow wheel coaxial-confocal attachment (Technical Instruments, San Francisco, CA, U.S.A.). Cells were mounted in a perfusion chamber and viewed with a Nikon Plan-Apo X60 oil immersion chamber objective (numerical aperture, 1.4). Confocal fluorescence images were detected with a cooled CCD camera (Micromax; Princeton Instruments, Evry, France) with a 12-bit detector (RTEA-1317 K; Kodak). $C_6$-NBD-ceramide or LysoTracker™ Green DND-26 and CO-1 were visualized with standard fluorescein and rhodamine filter sets, respectively. Image pairs were acquired (exposure time, 500 ms) for the same field containing one or more cells. Analysis and display were performed using IPLab software (Scanalytics, Fairfax, VA, U.S.A.).

### Lipophilicity measurements

HPLC column containing as stationary phase 1-myristoyl, 2-[13-carbonylimidazolide-tridecanoyl]-sn-3-glycerophospholine (lecithin-imidazolide) bonded to silica-propylamine with the unreacted propylamine moieties end-capped with C10 and C3 alkyl chains (IAM.PC.DD2) was purchased from Regis

Technologies Inc. (Morton Grove, Ill, U.S.A.). The IAM.PC.DD2 column was 3 cm × 4.6 mm; particle diameter 12 μm; pore diameter 300 Å. For all studies the injection volume was ca. 10 μl of a solute aqueous solution of anthrapyridone at concentration ~100 μM. Acetonitrile/0.1 M Sörensen buffer ($K_2HPO_4/KH_2PO_4$) pH 7.2 eluent was used in a proportion 50:50%, 35:65%, 25:75%, 20:80% and 15:85% (v $v^{-1}$). The flow-rate was 1 ml $min^{-1}$ and solute detection was at 495 nm. The chromatographic system consisted of a Model L-6200A pump, a Model L-4250 UV-VIS detector and a Model D-2500 chromatointegrator (all from Merck-Hitachi, Vienna, Austria).

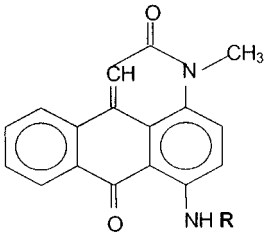
Capacity factors, $k'_{IAM}$, were calculated as $k'_{IAM} = t_r\text{-}t_0/t_0$. $t_r$ = retention time; $t_0$ = dead time of the column. The dead time of the column was the signal given by 50 μg/ml citric acid solution. A standard, commercially available statistical package for regression analysis was employed on a personal computer.

## Results

The structures of new synthesized anthrapyridones are presented in Figure 1. As can be seen the series of obtained compounds contained two types of anthrapyridones: (a) ANTHRAPYRIDONES OF TYPE I having hydrophobic substituents in the side chain: CO-1, CO-3, CO-5; (b) ANTHRAPYRIDONES OF TYPE II having hydrophylic substituents in the side chain: CO-4, CO-6, CO-7

### *Interaction of anthrapyridones with naked DNA*

In order to determine an affinity of examined compounds to isolated DNA, the fluorometric titration of anthrapyridones (1 μM) with naked DNA was performed at 37°C in HEPES buffer (pH 7.3). The addition of DNA to a compound solution yielded a quenching of the fluorescence intensity. For each compound, two parameters have been determined: $C_b$ – the concentration of anthrapyridone bound to DNA at the equilibrium state, $C_{DNA50}$ – the DNA concentration yielded 50% of the anthrapyridone binding $C_b$ (Table 2). As can be seen, anthrapyridones have a high affinity for DNA. This affinity was comparable for all examined derivatives as well as for reference compounds, doxorubicin and pirarubicin.




| R | |
|---|---|
| $CH_2CH_2N(CH_3)_2$ | C0-1 |
| $CH_2CH_2CH_2N(CH_3)_2$ | C0-3 |
| $CH_2CH_2NHC_2H_5$ | C0-4 |
| $CH_2CH_2N(C_2H_5)_2$ | C0-5 |
| $CH_2CH_2NHCH_2CH_2OH$ | C0-6 |
| $CH_2CH_2NH_2$ | C0-7 |

**Figure 1** Structures of anthrapyridone derivatives.

### *Accumulation of anthrapyridones in erythroleukemia K562 sensitive and K562/DOX resistant cells*

The accumulation of anthrapyridones has been studied comparatively in control cells and in energy-depleted cells. Figures 2 and 3 present the typical fluorescence signals observed when drug-sensitive and drug-resistant K562 cells were incubated in glucose-containing HEPES buffer respectively with 1 μM anthrapyridone of type I (CO-1, CO-3, CO-5) or anthrapyridone of type II (CO-4, CO-6, CO-7). For anthrapyridones of type I, very fast and important decrease of the fluorescence signal was observed with K562 sensitive as well as resistant cells due to the very rapid cellular uptake of anthrapyridone of type I (Figure 2). A steady state was obtained for these compounds within about 5 min. The concentration bound in the cells at the steady state was almost the same for sensitive and resistant lines and equal to 0.80 ± 0.05 μM. In contrast, for anthrapyridones of type II, very low decrease of fluorescence signal was observed with K562 sensitive and resistant cells due to the slow cellular uptake of these compounds. A steady state for derivatives of type II was obtained within about 1 h (Figure 3). Their accumulation in resistant cells was significantly reduced in comparison to sensitive cells mostly for anthrapyridones CO-6 and CO-7.

Figures 4 and 5 show the records of experiments performed respectively for anthrapyridones of type I and anthrapyridones of type II incubated with sensitive and resistant K562 cells in HEPES buffer in the presence of 10 mM $NaN_3$ and in the absence of glucose. The incorporation of anthrapyridones at the steady state in these energy-depleted cells was significantly modified in comparison to control cells. After the addition of 5 mM glucose giving rise to ATP synthesis *via* the glycolysis pathway, a further decrease of fluorescence signal was observed for anthrapyridones of type I, more distinct in the case of sensitive cells. For anthrapyridones of type II whereas, the addition of glucose to sensitive cells yielded to a slight decrease of fluorescence signal while for resistant cells an increase of this signal was observed. This is related to the occurrence of two

**Table 2** Affinity of anthrapyridones, doxorubicin and pirarubicin to naked DNA

| *Compound* | $C_b$ [μM] | $C_{DNA\ 50}$ [μM] |
|---|---|---|
| CO-1 | 0.80 | 4.6 |
| CO-3 | 0.86 | 5.6 |
| CO-4 | 0.73 | 5.7 |
| CO-5 | 0.75 | 4.8 |
| CO-6 | 0.72 | 4.8 |
| CO-7 | 0.66 | 5.4 |
| DOX | 0.92 | 2.6 |
| PIRA | 0.83 | 2.9 |

The reaction mixture (2 ml) contained: 1 μM examined compound, 0–72 μM DNA, 9.5 mM HEPES, 132 mM NaCl, 3.5 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.25. $C_b$ – the concentration of compound bound to DNA at the equilibrium state (±5%); $C_{DNA\ 50}$ – the DNA concentration yielded 50% of the compound binding $C_b$ (±5%).

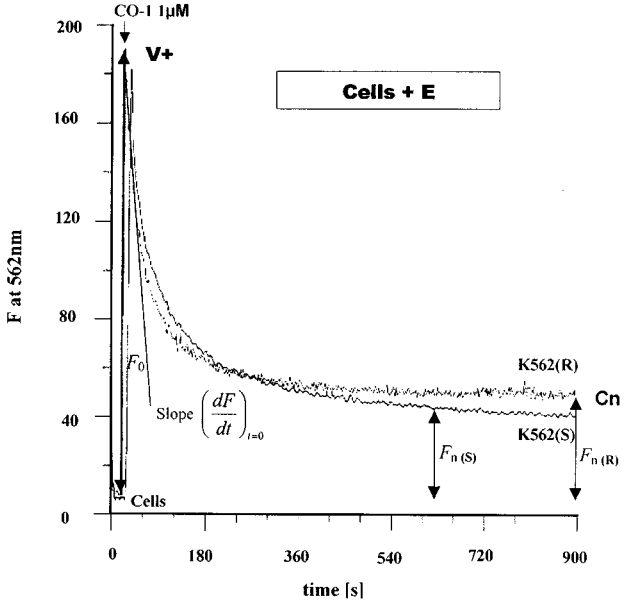



**Figure 2** Uptake of anthrapyridone of type I (CO-1) by sensitive (S) and resistant (R) K562 cells. Fluorescence intensity at 562 nm ($\lambda_{ex}=495$ nm) was recorded as a function of incubation time until the steady state. Cells, $2\times10^6$, either sensitive (S) or resistant (R), were suspended in a cuvette filled with 2 ml buffer at pH = 7.25 under vigorous stirring. At $t=0$, 20 $\mu$l of a 100 $\mu$M stock anthrapyridone solution was added to the cells yielding a $C_T=1$ $\mu$M anthrapyridone solution.

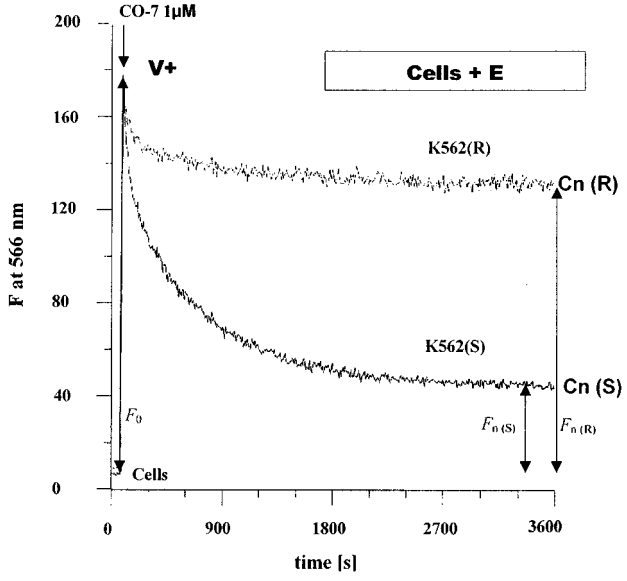



**Figure 3** Uptake of anthrapyridone of type II (CO-7) by sensitive (S) and resistant (R) K562 cells. Fluorescence intensity at 566 nm ($\lambda_{ex}=495$ nm) was recorded as a function of incubation time until the steady state. Cells, $2\times10^6$, either sensitive (S) or resistant (R), were suspended in a cuvette filled with 2 ml buffer at pH = 7.25 under vigorous stirring. At $t=0$, 20 $\mu$l of a 100 $\mu$M stock anthrapyridone solution was added to the cells yielding a $C_T=1$ $\mu$M anthrapyridone solution.

simultaneous energy-dependent processes: non-nuclear accumulation ($V_{nn}$) which causes an autoquenching of fluorescence signal due to the small volume of non-nuclear organelles and P-glycoprotein-mediated efflux ($V_{P\text{-}gp}$) which gives an increase of fluorescence signal due to the release of anthrapyridones out of the cells. It is possible to estimate the contribution of each of these processes because, for energy-depleted sensitive cells after the addition of glucose, only non-nuclear accumulation was observed ($V_{E(S)}=V_{nn(S)}$). It seems also very probable that the rate of non-nuclear accumulation limited by the step of the passive passage through intracellular membranes do not depend on cell line ($V_{nn(S)}\cong V_{nn(R)}$), since it was found that the kinetics of cellular uptake determined by the step of a passive passage of anthrapyridone through the plasma membrane was the same for sensitive and resistant cells. Taking into account these assumptions, the P-glycoprotein-mediated efflux contribution in the summary rate of energy dependent processes $V_{E(R)}$ can be calculated using following expressions:

For anthrapyridones of type I:
- in the case of sensitive cells: $V_{E/(S)}=V_{nn(S)}$
- in the case of resistant cells: $V_{E(R)}=V_{nn(R)}\text{-}V_{P\text{-}gp}$, $V_{nn(S)}\approx V_{nn(R)}$, $V_{nn}>V_{P\text{-}gp}$ $V_{P\text{-}gp}=V_{E(S)}\text{-}V_{E(R)}$

For anthrapyridones of type II:
- in the case of sensitive cells: $V_{E(S)}=V_{nn(S)}$
- in the case of resistant cells: $V_{E(R)}=V_{P\text{-}gp}\text{-}V_{nn(R)}$, $V_{nn(S)}\approx V_{nn(R)}$, $V_{P\text{-}gp}>V_{nn}$ $V_{P\text{-}gp}=V_{E(S)}+V_{E(R)}$

Additional studies have been done with verapamil, a well-known P-glycoprotein blocking agent to support the existence of P-gp-mediated efflux of anthrapyridones. We have found that 10 $\mu$M verapamil inhibited the efflux of these compounds to about 70% (to the same extent that for reference compound pirarubicin, 75%) (data not presented).

Figure 6 and Figure 8b show diagrams representing anthrapyridone accumulation in control (cells + E) and in energy deprived (cells-E) K562 cells. Figure 7 and Figure 8a show comparative diagrams representing the values of kinetics of passive cellular uptake, kinetics of active P-gp-mediated efflux and the rate of non-nuclear accumulation for all examined anthrapyridones.

### *Intracellular distribution of anthrapyridones in NIH-3T3 cells*

The above data showed that, beside an energy-independent accumulation of anthrapyridones in nucleus, an energy-dependent accumulation of these compounds occurred in non-nuclear compartments. In order to define the intracellular distribution of anthrapyridones, NIH-3T3 cells were used, because they have relatively small nucleus and thus their intracellular organelles are easy visualized. Cells were incubated with 1 $\mu$M CO-1 and then with LysoTracker™ Green DND-26 to stain lysosomes or with $C_6$-NBD-ceramide-bovine serum albumin complex to stain trans-Golgi. The series of images obtained by confocal microscopy (Figure 9) showed the accumulation of anthrapyridones in nucleus as well as punctuated zones of strong fluorescence signal have been detected. Confocal microscopy analysis using Lyso-Tracker™ Green DND-26, the specific probe for lysosomes, showed that these punctuated zones corresponded to the lysosomal accumulation. Additional confocal microscopy

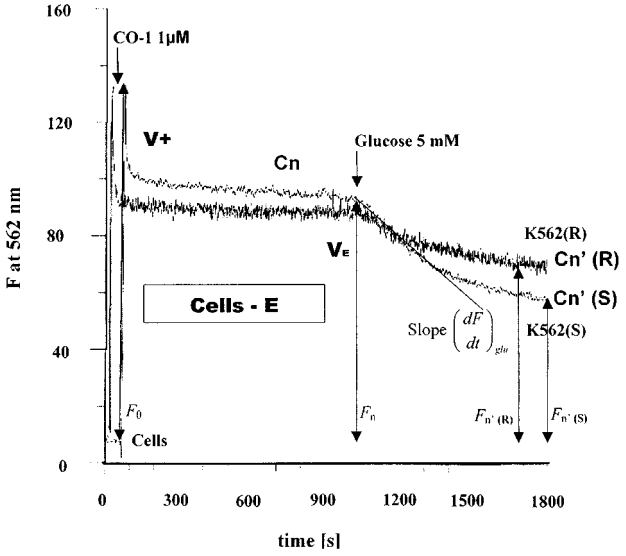



**Figure 4** Uptake of anthrapyridone of type I (CO-1) by energy-deprived sensitive (S) and resistant (R) K562 cells. Fluorescence intensity at 526 nm ($\lambda_{ex}=495$ nm) was recorded as a function of incubation time. Cells, $2\times10^6$, either sensitive (S) or resistant (R), were suspended in a cuvette filled with 2 ml buffer at pHe = 7.25 under vigorous stirring and preincubated for 30 min with 10 mM $NaN_3$ in the absence of glucose. At $t=0$, 20 $\mu$l of a 100 $\mu$M stock anthrapyridone solution was added to the cells yielding a $C_T=1$ $\mu$M anthrapyridone solution. When the steady state was reached, 5 mM glucose was added yielding a new steady state. The global rate of energy-dependent processes ($V_E$) was determined from the slope of the tangent to the curve after the addition of glucose.

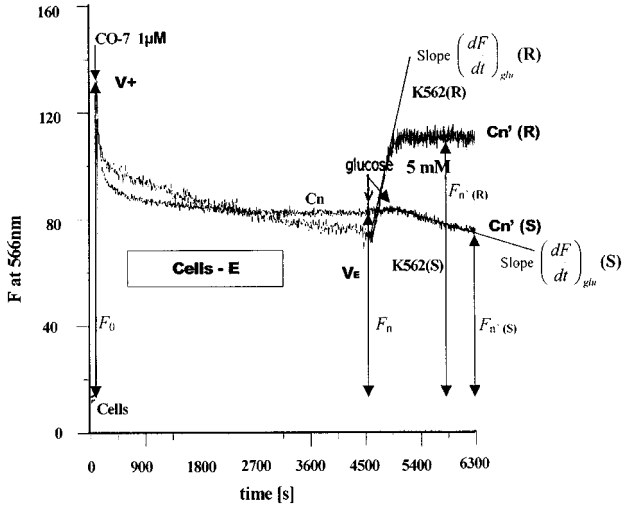



**Figure 5** Uptake of anthrapyridone of type II (CO-7) by energy-deprived sensitive (S) and resistant (R) K562 cells. Fluorescence intensity at 566 nm ($\lambda_{ex}=495$ nm) was recorded as a function of incubation time. Cells, $2\times10^6$, either sensitive (S) or resistant (R), were suspended in a cuvette filled with 2 ml buffer at pHe = 7.25 under vigorous stirring and preincubated for 30 min with 10 mM $NaN_3$ in the absence of glucose. At $t=0$, 20 $\mu$l of a 100 $\mu$M stock anthrapyridone solution was added to the cells yielding a $C_T=1$ $\mu$M anthrapyridone solution. When the steady state was reached 5 mM glucose was added yielding a new steady state. The global rate of energy-dependent processes ($V_E$) was determined from the slope of the tangent to the curve after the addition of glucose.

analysis done with trans-Golgi probe ($C_6$-NBD-ceramide-bovine serum albumin complex) showed that there was no accumulation of anthrapyridones in trans-Golgi organelles. The lysosomal accumulation of anthrapyridones being weak bases is related to an energy-dependent pH gradient and was observed in our transport studies as an energy-dependent accumulation. From all these data, we can infer that lysosomes are a second site of anthrapyridones accumulation.

### *Determination of lipophilic properties of anthrapyridones*

Measurements of lipophilicity parameters of anthrapyridones have been done by RF-HPLC method using an immobilized artificial membrane column (IAM). This method seems to be more accurate in comparison to standard measure of lipophilicity parameters as a partition coefficient in n-octanol/buffer system (Kaliszan *et al*., 1994).

For all examined anthrapyridones, the capacity factors $k'_{IAM}$ ($k'_{IAM}=t_r\text{-}t_o/t_o$; $t_r$- retention time, $t_o$-dead time of the column) have been determined. It was found that there was nearly a linear correlation between the $lgk'_{IAM}$ value of this lipophilicity parameters and $lgV_+$ as well as between $lgk'_{IAM}$ and $lgk_{nn}$ (were: $k_{nn}$-coefficient of non-nuclear accumulation rate $k_{nn}=V_{nn}/C_i$) (Figure 10).

### *Cytotoxic activity of anthrapyridones*

The anthrapyridone concentrations required for a 50% inhibition ($IC_{50}$) of respectively sensitive and resistant K562 cell growth are shown in Table 3 with the resistance factor (RF) values. For comparison, the values obtained for doxorubicin and pirarubicin have been added. As can be seen, for anthrapyridones of type I the RF values are low (1.1 ÷ 2.8) while for anthrapyridones of type II the significant decrease of cytotoxic activity against K562 resistant cells was observed. The RF values for CO-4 and for CO-6 and CO-7, the less active derivatives against K562 resistant cells, were respectively 6, 67 and 34. These *in vitro* results corroborate with the accumulation data presented above. The decrease in accumulation of CO-4 in resistant cells was much less important (47%) than for CO-6 (88%) and CO-7 (73%) (Figure 8b). We have found that nearly linear relation exists between the resistance factor RF and the parameter $C_n(S)/C_n(R)$ values reflecting the decrease in anthrapyridones accumulation in resistant cells in comparison to sensitive parent line ($r=0.976$) (Figure 11). Although the resistance factors for anthrapyridone of type I were near 1, they exhibited relatively low cytostatic activity on sensitive cells in comparison to doxorubicin and pirarubicin.

## Discussion

The rational design of antitumour agents able to circumvent multidrug resistance is one of the most important questions for an effective cancer therapy. The reduced drug accumulation, due to the active export of cytostatics out of resistant cells is the main cause of multidrug resistance (Garnier-Suillerot, 1995).

Our approach to search for non-cross resistant antitumour agents is based on the fact that the intracellular drug concentration attainable in resistant cells depends on the

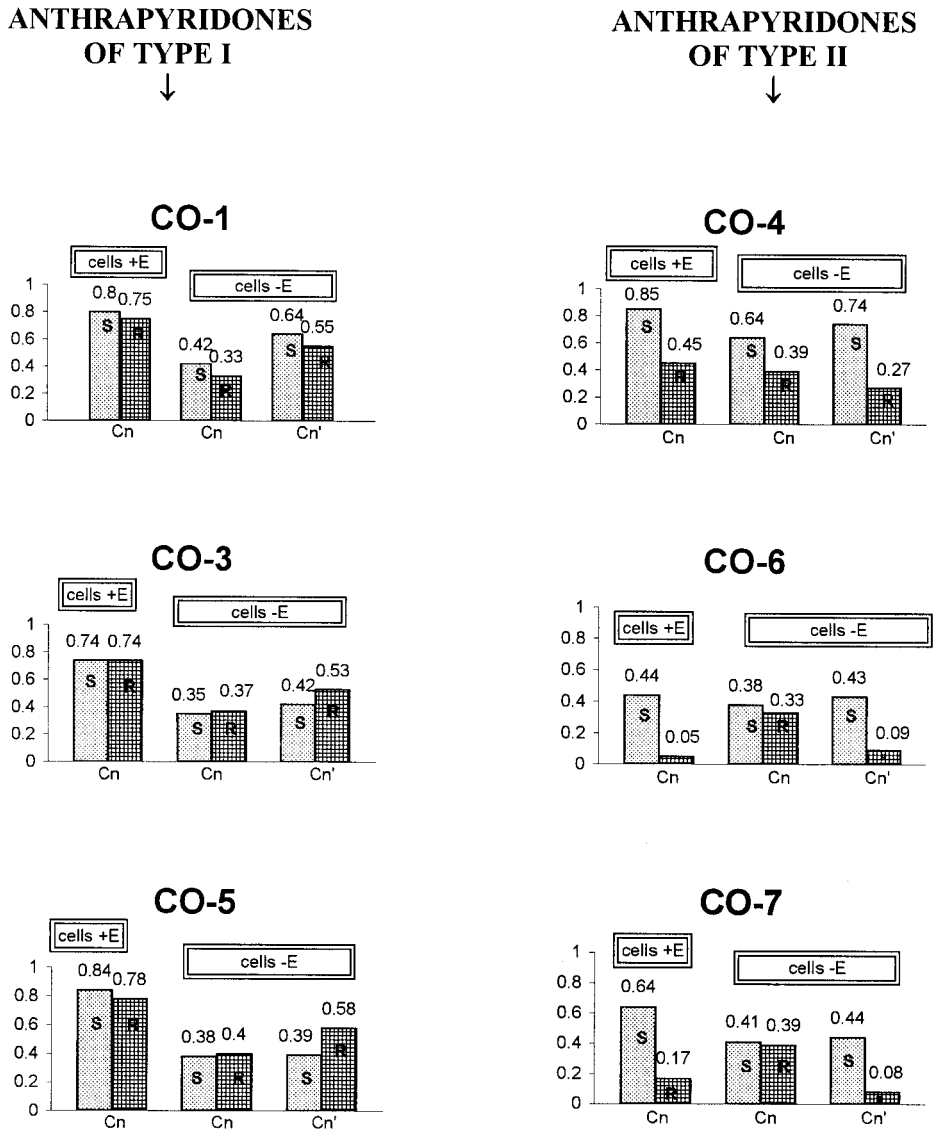



**Figure 6** Anthrapyridone accumulation in control (cells + E) and energy-deprived (cells-E) K562 cells. $C_n$ = anthrapyridone concentration bound in the cells at the steady state; $C'_n$ = anthrapyridone concentration bound in the cells at the new steady state after the addition of 5 mM glucose to energy-deprived cells. The values are given in $\mu$M ($\pm$10%). $C_T$ = total anthrapyridone concentration; $C_T$ = 1 $\mu$M; S-sensitive cells; R-resistant cells.

difference between the kinetics of passive cellular uptake and kinetics of active ABC transporter-mediated efflux (Mankhetkorn *et al*., 1996; Marbeuf-Gueye *et al*., 1998; 1999). It might be possible to design derivatives which, for the reason of their physicochemical properties, diffuse very rapidly into the cell and in such cases the protein transporter responsible for the effective drug export out of the cell essentially operates in a futile cycle. We have recently demonstrated that benzoperimidines (Stefaska *et al*., 1999; Tkaczyk-Gobis *et al*., 2001) and properly substituted pyrimidoacridones (Antonini *et al*., 1995), exhibiting very high kinetics of cellular uptake, were able to overcome multidrug resistance mediated either by P-gp or MRP1. We postulate further that the presence of 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 of obtained derivatives across the plasma membrane. In order to further support this hypothesis, a novel family of anthrapyridones was obtained and examined in regard to erythroleukemia K562 sensitive and K562/DOX resistant cells with the overexpression of P-glycoprotein. Anthrapyridones, carboquinoid derivatives of anthracenediones, contain an additional six-membered ring condensed to the anthracenedione chromophore. A series of six compounds was properly designed. Anthrapyridones of type I have hydrophobic substituents on the side chain favouring *a priori* the kinetics of cellular uptake and anthrapyridones of type II, designed as a negative control, have hydrophylic substituents on the side chain disturbing *a priori* the kinetics of cellular uptake. The data presented in this paper indicate that: (i) all examined anthrapyridones have a high affinity to naked DNA; (ii) the modulation of the kinetics of passive cellular uptake of anthrapyridones $V_+$ is much easier to perform by suitable structure modifications of the side chain. The kinetics of P-gp-dependent active efflux of anthrapyridones $V_{P\text{-}gp}$ is the parameter much less susceptible to modulate by

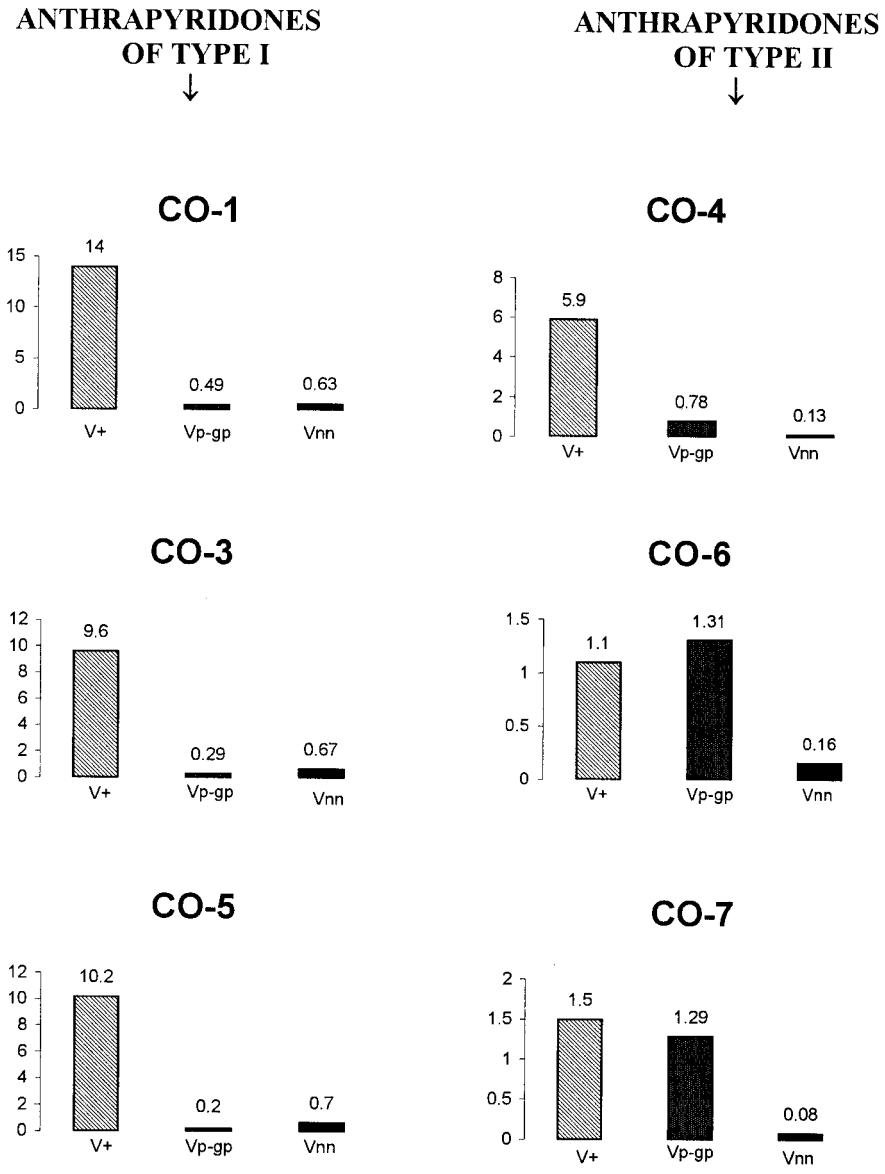



**Figure 7** Diagrams representing the values of $V_+$, $V_{P\text{-}gp}$ and $V_{nn}$ for anthrapyridone derivatives: $V_+$ = kinetics of passive cellular uptake; $V_{P\text{-}gp}$ = kinetics of active P-gp-mediated efflux; $V_{nn}$ = rate of non-nuclear accumulation. The values are given in $10^{-18}$ mole cell$^{-1}$ s$^{-1}$ ($\pm$10%).

chemical modifications, pointing to the similarity of the substrate spectrum of all tested compounds for P-gp; (iii) introduction of hydrophobic substituents to the side chain of anthrapyridones [CO-1, CO-3, CO-5] favours the fast passive cellular uptake ($V_+ >> V_{P\text{-}gp}$) (Figure 8a) and causes that their cellular accumulation determining the cytotoxic activity are very high and comparable for sensitive and resistant cells ($C_{n(R)} \approx C_{n(S)}$) (Figure 8b); (iv) introduction of hydrophylic substituents to the side chain of anthrapyridones [CO-4, CO-6, CO-7] disturbs the fast passive cellular uptake ($V_+ \approx V_{P\text{-}gp}$) (Figure 8a) and causes a drastic decrease of cellular accumulation in resistant cells ($C_{n(R)} < C_{n(S)}$) (Figure 8b); (v) cellular accumulation of anthrapyridones occurs in the nucleus, probably through intercalation of the flat conjugated rings between the bases pairs. Our data show that these compounds being weak bases accumulate also in lysosomes in an energy-dependent manner; (vi) a nearly linear relation exists between lipophilicity parameter $lgk'_{IAM}$ and $lgV_+$ as well as between $lgk'_{IAM}$ and $lgk_{nm}$ corroborating the very important role of the hydrophobic-hydrophylic character of examined anthrapyridones in their ability to cross biological membranes (cytoplasmic and membranes surrounding cellular organelles). In contrast, it seems that the hydrophobic-hydrophylic character of anthrapyridones does not determine their affinity for P-gp exporting pump.

It is well demonstrated for anthracyclines that there is a good correlation between the short-term measurements of drug accumulation in living cells and the long-term growth inhibition (Pereira & Garnier-Suillerot, 1994; Garnier-Suillerot, 1995). Our *in vitro* biological data confirm that anthrapyridones of type I conserving very high accumulation in resistant cells exhibit comparable cytotoxic activity against K562 sensitive and K562/DOX resistant cells, while anthrapyridones of type II are much less active against K562/DOX resistant cells in comparison to sensitive cells.

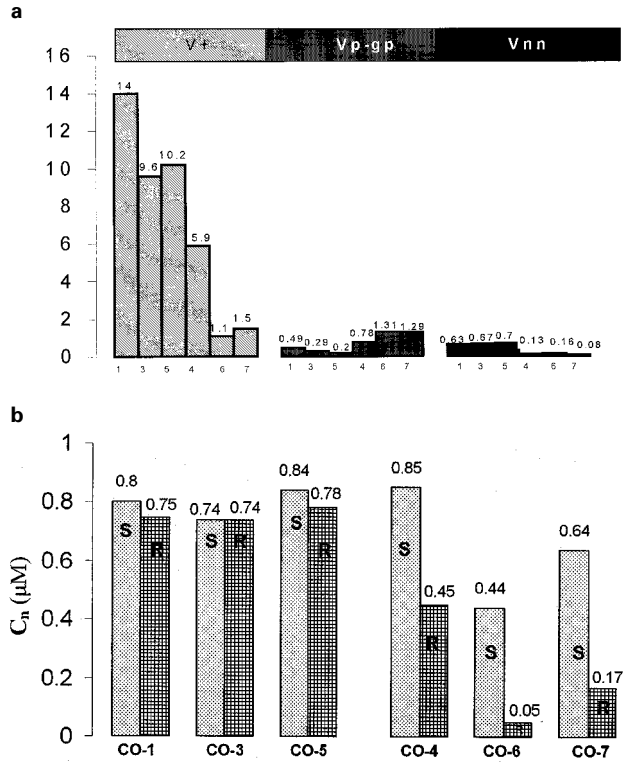



**Figure 8** Comparative diagrams representing: (a) the values of $V_+$, $V_{P\text{-}gp}$ and $V_{nn}$ for anthrapyridone derivatives; $V_+$ = kinetics of passive cellular uptake; $V_{P\text{-}gp}$ = kinetics of active P-gp-mediated efflux; $V_{nn}$ = rate of non-nuclear accumulation; The values are given in $10^{-18}$ mole cell$^{-1}$ s$^{-1}$ ($\pm$10%): 1 – compound CO-1, 3 – compound CO-3, 5 – compound CO-5, 4 – compound CO-4, 6 – compound CO-6, 7 – compound CO-7. (b) the intracellular anthrapyridone accumulation ($C_n$) in sensitive (S) and resistant (R) K562 cells; $C_n$ = anthrapyridone concentration bound in the cells at the steady state. The values are given in $\mu$M ($\pm$10%). $C_T$ = total anthrapyridone concentration; $C_T$ = 1 $\mu$M.

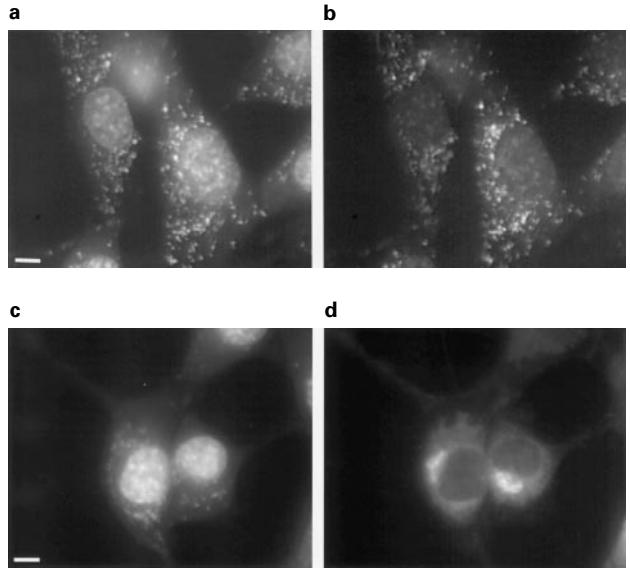



**Figure 9** Colocalization of CO-1 with lysosomes and trans-Golgi probes in NIH/3T3 cells. Cells were labelling according to the procedure described in Methods with 1 $\mu$M CO-1 (A and C), specific lysosome probe: LysoTracker™ Green DND-26 (B), specific trans-Golgi probe: $C_6$-NBD-ceramide-bovine serum albumin complex (D).

Our preliminary results done on small-cell lung cancer cell line GLC4/DOX overexpressing MRP1 show that anthrapyridones of type I exhibit also comparable cytotoxic activity towards sensitive and MRP1 resistant cells, while for reference compound – doxorubicin RF(GLC4) is equal to 74. The presented results show that it is possible to design non-cross resistant antitumour agents, simply by the fact that they penetrate very rapidly into the cell. The confirmation of this postulate was the main aim of this study. Although the resistance factors for anthrapyridones of type I are near 1, it seems hardly probable that they could find a clinical application as potential cytostatics in the treatment of resistant types of cancers because of their relatively low cytostatic activity on sensitive cells. In fact, the cytotoxic activity of anticancer agents depends on intracellular concentration and for resistant cells is determined by the kinetics of cellular uptake and kinetics of an energy-

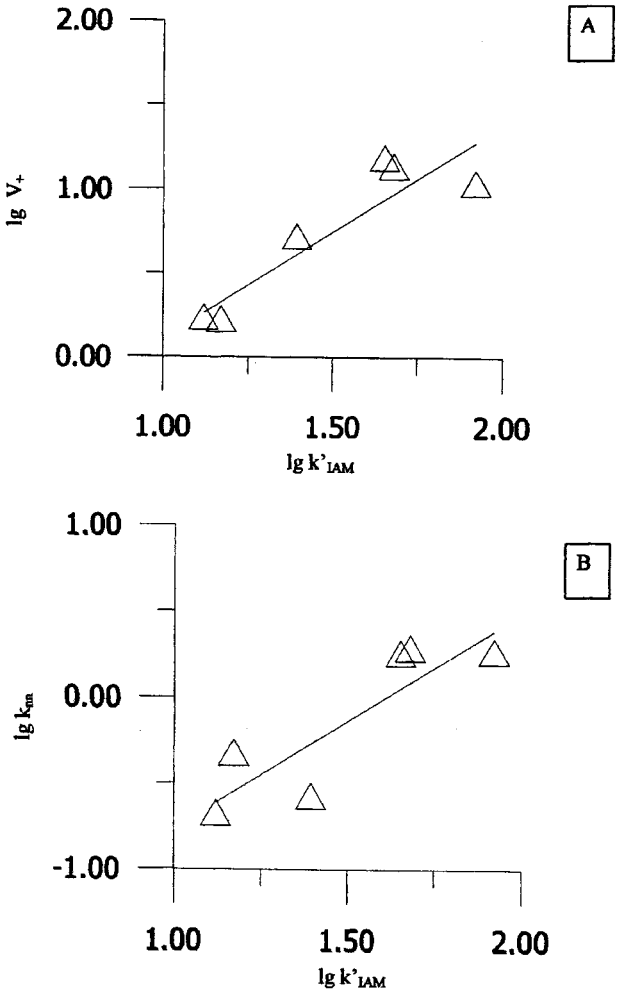



**Figure 10** Dependence of $IgV_+$ and $lgk_{nn}$ of anthrapyridone compounds on the lipophilicity parameter $lgk'_{IAM}$: $V_+$ = kinetics of passive cellular uptake; $k_{nn}$ = coefficient of non-nuclear accumulation rate $k_{nn} = V_{nn}/C_i$: $V_{nn}$ – rate of non-nuclear accumulation, $C_i$ = free anthrapyridone concentration in the cytoplasm of energy-deprived (cells-E), K562 cells at the steady state, $C_i = C_T - C_n$. $k'_{IAM}$ = the capacity factor measured on an immobilized artificial membrane column IAM ($k'_{IAM} = t_r - t_0/t_0$; $t_r$-retention time, $t_0$ – dead time of the column). The lines drawn have been least-squares fitted to the data. The correlation coefficients are higher than 0.92.

**Table 3** Cross-resistance pattern of doxorubicin-resistant K562 cells

| *Compound* | *K562* $IC_{50}(S)[\mu M]$ | *K562/DOX* $IC_{50}(R)[\mu M]$ | *RF* |
|---|---|---|---|
| CO-1 | 0.7±0.2 | 2.0±0.5 | 2.8 |
| CO-3 | 11.0±0.1 | 12.0±0.1 | 1.1 |
| CO-5 | 0.8±0.3 | 2.1±0.2 | 2.6 |
| CO-4 | 0.5±0.1 | 3.0±0.2 | 6.0 |
| CO-6 | 0.95±0.04 | 64±9 | 67 |
| CO-7 | 1.8±0.1 | 62±12 | 34 |
| DOX | 0.010±0.002 | 0.34±0.03 | 34 |
| PIRA | 0.006±0.001 | 0.050±0.003 | 8 |

$IC_{50}(S)$ and $IC_{50}(R)$ are the compound concentrations required to inhibit 50% of respectively sensitive and resistant cell growth. Resistance factor (RF) was calculated as $RF = IC_{50}(R)/IC_{50}(S)$. The values represent mean + s.d. of triplicate determinations.

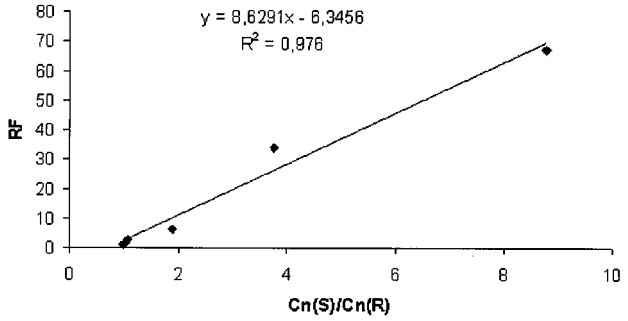



**Figure 11** Dependence of resistance factor RF on the $C_n(S)/C_n(R)$ parameter for anthrapyridones compounds. The values $C_n(S)/C_n(R)$ reflect the decrease in anthrapyridones accumulation at the steady state in resistant cells $C_n(R)$ in comparison to sensitive parent line $C_n(S)$.

dependent export. Nevertheless, the cytotoxic activity depends highly on mechanism(s) of interaction of anticancer agents with DNA machinery (DNA intercalation, alkylation or cross-linking mechanisms, inhibition of topoisomerase I and/or II etc). The examination of these mechanisms could be the subject of another study in a further step. In this stage of our work we can only suggest that the relatively low cytostatic activity of anthrapyridones against sensitive cells would be related in part to their accumulation not only in nucleus (as in the case of doxorubicin) but also in lysosomes. Nevertheless, presented data suggest that: *firstly* – non-nuclear accumulation of cytostatics is not necessarily very toxic for the cell and in consequence do not exclude their clinical use; *secondly* – we consider the potential application of anthrapyridones of type I as effective modulators of multidrug resistance exporting pumps which, in the presence of these derivatives, would operate in a futile cycle mode. It creates the possibility to reverse the multidrug-resistance to anthracycline antitumour agents by the combinated administration of these drugs with anthrapyridone compounds. Studies on the ability of anthrapyridones to inhibit the P-gp and MRP1-dependent export of anthracyclines and in consequence to restore their cytotoxic activity against K562/DOX and GLC4/DOX resistant cell lines is under progress in our laboratory.

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## References

ALAHARI, S.K., DEAN, N.M., FISHER, M.H., DELONG, R., MANOHARAN, M., TIVEL, K.L. & JULIANO, R.L. (1996). Inhibition of expression of the multidrug resistance-associated P-glycoprotein by phosphorothioate and 5′-cholesterol-conjugated phosphorothioate antisense oligonucleotides. *Mol. Pharmacol.,* **50,** 808–819.

ANTONINI, I., COLA, D., POLUCCI, P., BONTEMPS-GRACZ, M., BOROWSKI, E. & MARTELLI, S. (1995). Synthesis of (dialkylamino)alkyl-disubstituted pyrimido[5,6,1-*de*]acridines, a novel group of anticancer agents active on a multidrug resistant cell line. *J. Med. Chem.,* **38,** 3282–3286.

BASSAN, R., CHIODINI, B., LEREDE, T., TORRI, V., BORLERI, G. & RAMBALDI, A. (1997). The role of idarubicin in adult acute lymphoblastic leukemia: From drug resistance studies to clinical application Burbui, T. *Leukemia,* **26,** 89–97.

BECK, W.T. (1987). The cell biology of multiple drug resistance. *Biochem. Pharmacol.,* **18,** 2879–2887.

BERGER, D., CITARELLA, R., DUTIA, M., GREENBERGER, L., HALLETT, W., PAUL, R. & POWELL, D. (1999). Novel multidrug resistance reversal agents. *J. Med. Chem.,* **42,** 2145–2161.

CHAUDHARY, P.M. & RONINSON, L.B. (1993). Induction of multidrug resistance in human cells by transient exposure to different chemotherapeutic drugs. *J. Natl. Cancer Inst.,* **85,** 632–639.

DOYLE, L.A., GORO, Y.M., YONG, W.D. & ROSS, D.D. (1995). Characterization of a 95 kilodalton membrane glycoprotein associated with multi-drug resistance. *Int. J. Cancer,* **62,** 588–593.

FREZARD, F. & GARNIER-SUILLEROT, A. (1991). Comparison of the membrane transport of anthracycline derivatives in drug-resistant and drug-sensitive K562 cells. *Eur. J. Biochem.,* **196,** 483–491.

GAJ CHL, ANYANWUTAKU, I., YU HUA CHANG & YUNG-CHI CHENG. (1998). Decreased drug accumulation without increased drug efflux in a novel MRP-overexpressing multidrug-resistant cell line. *Biochem. Pharmacol.,* **55,** 1199–1211.

GARCIA-CHAUMONT, CH., SEKSEK, O., JOLLES, B. & BOLARD, J. (2000). A cationic derivative of amphotericin B as a novel delivery system for antisense oligonucleotides. *Antisense Nucl. Acid Dev.,* **10,** 177–184.

GARNIER-SUILLEROT, A. (1995). Impaired accumulation of drug in multidrug resistant cells. What are the respective contributions of the kinetics of uptake and of P-glicoprotein-mediated efflux of drug? *Curr. Pharm. Des.,* **1,** 69–82.

GERMANN, U.A. (1996). P-Glycoprotein – a mediator of multidrug resistance in tumour cells. *Eur. J. Cancer,* **32,** 927–944.

GOTTESMAN, M.M. & PASTAN, L. (1993). Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu. Rev. Biochem.,* **62,** 385–427.

KALISZAN, R., NASAL, A. & BUCIŃSKI, A. (1994). Chromatographic hydrophobicity parameter determined on an immobilized artificial membrane column: relationships to standard measures of hydrophobicity and bioactivity. *Eur. J. Med. Chem.,* **29,** 163–170.

KOOL, M., DE HAAS, M., SCHEFFER, G.L., SCHEPER, R.J., VAN EIJK, M.J.T., JUIJN, J.A., BAAS, F. & BORST, P. (1997). Analysis of expression of cMOAT (MRP2), MRP3, MRP4 and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1) in human cancer cell lines. *Cancer Res.,* **57,** 3537 – 3547.

KUBOTA, T., FURUKAWA, T., TANINO, H., OURA, S., MURATA, H., YUASA, S., MORITA, K., UENO, J., KOZAKAI, R. & YANO, T. (1998). Pirarubicin might partly circumvent the P-glycoprotein-mediated drug resistance of human breast cancer tissues. *Anti-Cancer Res.,* **18,** 967 – 972.

LOE, D.W., DEELEY, R.G. & COLE, S.P.C. (1996). Biology of the multidrug resistant-associated protein, MRP. *Eur. J. Cancer,* **32,** 945 – 957.

MANKHETKORN, S., DUBRU, F., HESSCHENBROUCK, J., FIALLO, M. & GARNIER-SUILLEROT, A. (1996). Relation among the resistance factor, kinetics of uptake and kinetics of the P-glycoprotein-mediated efflux of doxorubicin, daunorubicin, 8-(S)-fluoroidarubicin, and idarubicin in multidrug-resistant K562 cells. *Mol. Pharmacol.,* **49,** 532 – 539.

MANKHETKORN, S. & GARNIER-SUILLEROT, A. (1996). The ability of verapamil to restore intracellular accumulation of anthracyclines in multidrug resistant cells depend on the kinetics of their uptake. *Eur. J. Biochem.,* **343,** 313 – 321.

MARBEUF-GUEYE, C., BROXTERMAN, H.J., DUBRU, F., PRIEBE, W. & GARNIER-SUILLEROT, A. (1998). Kinetics of anthracycline efflux from multidrug resistance protein-expressing cancer cells compared with P-glycoprotein-expressing cancer cells. *Mol.. Pharmacol.,* **53,** 141 – 147.

MARBEUF-GUEYE, C., ETTORI, D., PRIEBE, W., KOZOWSKI, H. & GARNIER-SUILLEROT, A. (1999). Correlation between the kinetics of anthracycline uptake and the resistance factor in cancer cells expressing the multidrug resistance protein or the P-glycoprotein. *Biochim. Biophys. Acta,* **1450,** 1 – 11.

PALFNER, K., KNEBA, M., HIDDEMAN, W. & BERTRAM, J. (1995). Improvement of hammerhead ribozymes cleaving mdr-1 mRNA. *Biol. Chem.,* **376,** 289 – 295.

PASCAUD, C., GARRIGOS, M. & ORLOWSKI, S. (1998). Multidrug resistance transporter P-glycoprotein has distinct but interacting binding sites for cytotoxic drugs and reversing agents. *Biochem. J.,* **333,** 351 – 358.

PANAGO, R.E., SEPANSKI, M.A. & MARTIN, O.C. (1989). Molecular trapping of a fluorescent ceramide analogue at the Golgi apparatus of fixed cells: interaction with endogenous lipids provides a trans-Golgi marker for both light and electron microscopy. *J. Cell Biol.,* **109,** 2067 – 2079.

PAUL, S., BREUNINGER, L.M., TEW, K.D., SHEN, H. & KRUH, G.D. (1996). ATP-dependent uptake of natural product cytotoxic drugs by membrane vesicles establishes MRP as a broad specifity transporter. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 6929 – 6934.

PEREIRA, E., BORREL, M.N., FIALLO, M. & GARNIER-SUILLEROT, A. (1994). Non-competitive inhibition of P-glycoprotein-associated efflux of THP-adriamycin by verapamil in living K562 leukemia cells. *Biochim. Biophys. Acta,* **1225,** 209 – 216.

PEREIRA, E. & GARNIER-SUILLEROT, A. (1994). Correlation between the short-term measurements of drug accumulation in living cells and the long-term growth inhibition. *Biochem. Pharmacol.,* **47,** 1851 – 1857.

RADERER, M. & SCHEITHANER, W. (1993). Clinical trials of agents that reverse multidrug resistance. A literature review. *Cancer,* **72,** 3553 – 3563.

RONINSON, I.B. (1992). The role of the MDR1 (P-glycoprotein) gene in multidrug resistance in vitro and in vivo. *Biochem. Pharmacol.,* **43,** 95 – 102.

SCHEFFER, G.L., WIJNGAARD, P.L.J., FLEUS, M.J., IZGUIERDO, M.A., SLOVAK, M.L., PINEDO, H.M., MEIJER, C.J.L., CLEVERS, H.C. & SCHEPER, R.J. (1995). The drug resistance related protein LRP is the human major vault protein. *Nat. Med.,* **1,** 578 – 582.

STEFAŃSKA, B., DZIEDUSZYCKA, M., BONTEMPS-GRACZ, M., BOROWSKI, E., MARTELLI, S., SUPINO, R., PRATESI, G., DI CESARE, A., ZUNINO, F., KUŚNIERCZYK, H. & RADZIKOWSKI, CZ. (1999). 8,11-dihydroxy-6-[(aminoalkyl)amino]-7H-benzo[e]perimidin-7-ones with activity in multidrug resistant cell lines. Synthesis and antitumor evaluation. *J. Med. Chem.,* **42,** 3494 – 3501.

STEWART, A.J., CANITROT, Y., BARACCHINI, E., DEAN, N.M., DEELEY, R.G. & COLE, S.P.C. (1996). Reduction of expression of the multidrug resistance protein (MRP) in human tumor cells by antisense phosphorothioate oligonucleotides. *Biochem. Pharmacol.,* **51,** 461 – 469.

TARASIUK, J., FREZARD, F., GARNIER-SUILLEROT, A. & GATTEGNO, L. (1989). Interaction of anthracyclines with human lymphocytes. Kinetics of drug incorporation. *Biochim. Biophys. Acta,* **1013,** 109 – 117.

TEODORI, E., DEI, S., QUIDU, P., BUDRIESI, R., CHIARINI, A., GARNIER-SUILLEROT, A., GUALTIERI, F., MANETTI, D., ROMANELLI, M.N. & SCAPECCHI, S. (1999). Design, synthesis, and in vitro activity of catamphilic reverters of multidrug resistance (MDR). The discovery of a selective, highly efficacious chemosensitizer with potency in the nanomolar range. *J. Med. Chem.,* **42,** 1687 – 1697.

TKACZYK-GOBIS, K., TARASIUK, J., SEKSEK, O., STEFAŃSKA, B., BOROWSKI, E. & GARNIER-SUILLEROT, A. (2001). Transport of new non-cross resistant antitumor compounds of benzoperimidine family in multidrug resistant cells. *Eur. J. Pharmacol.,* **413,** 131 – 141.

TSURUO, T., SAITO, H.J., KAWABATA, H., OH-HARA, T., HAMADA, H. & UTAKOJI, T. (1986). Characteristics of resistance to adriamycin in human myelogenous leukemia K562 resistant to adriamycin and isolated clones. *Jpn. J. Cancer Res.,* **77,** 682 – 692.

ZAMAN, G.J.R., FLENS, M.J., VAN LEUSDEN, M.R., DE HAAS, M., MÜLDER, H.S., LANKELMA, J., PINEDO, H.M., SCHEPER, R.J., BASS, F., BROXTERMAN, H.J. & BORST, P. (1994). The human multidrug resistance-associated protein MRP is a plasma membrane drug-efflux pump. *Proc. Natl. Acad. Sci. U.S.A.,* **91,** 8822 – 8826.

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