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# Membrane effects of the antitumor drugs doxorubicin and thaliblastine: comparison to multidrug resistance modulators verapamil and *trans*-flupentixol

Ilza Pajeva [a,*], Dimiter K. Todorov [b], Joachim Seydel [c,1]

[a] *Centre of Biomedical Engineering, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 105, 1113 Sofia, Bulgaria*
[b] *Department of Oncopharmacology, National Centre of Oncology, 1156 Sofia, Bulgaria*
[c] *Research Center Borstel, Parlallee 1-40, 23845 Borstel, Germany*

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**Abstract**

The interactions of the antitumor drugs doxorubicin and thaliblastine with model membranes composed of neutral (phosphatidylcholine) and negatively charged (phosphatidylserine) phospholipids were studied by differential scanning calorimetry and nuclear magnetic resonance. The membrane activities of doxorubicin and thaliblastine were compared to those of the powerful multidrug resistance (MDR) modulators *trans*-flupentixol and verapamil. The results point out to the potential role of the drug–membrane interactions for the effects of doxorubicin and thaliblastine in resistant tumor cells. They direct also to the artificial membranes as a suitable tool for screening of compounds with potential ability to modulate MDR.


*Keywords:* Doxorubicin; Thaliblastine; MDR; MDR modulators; Drug–membrane interactions

## 1. Introduction

Multidrug resistance (MDR) in tumor cells is mainly associated with decreased intracellular accumulation of the cytotoxic agent as a result of its increased efflux by the ATP-dependent membrane-bound transport P-glycoprotein (P-gp) (Ambudkar et al., 1999). The anthracycline antibiotic doxorubicin (DOX) (Fig. 1) belongs to the anticancer drugs that are able to elicit MDR. Although the interaction of DOX with DNA- and DNA-associated enzymes is well recognized (Bouma et al., 1986), the drug is reported to exert a cytotoxic effect also through interaction with the cell membrane, especially with negatively charged phospholipids (Goormaghtigh et al., 1980; Triton and Yee, 1982; Nicloay et al., 1988). Increase in DOX binding to liposomes and membranes at increasing content of the anionic phosphatidylglycerol is observed (De Wolf et al., 1993). DOX binding and insertion into the membrane is shown to affect the intrinsic transport characteristics of the membrane (Speelmans et al., 1994). Resistant cells without over-expression of P-gp are found to decrease preferentially the content of DOX in the lipid fraction of the membrane as compared to the whole cell (Awasthi et al., 1992). It is also supposed that modulation of DOX toxicity by verapamil may involve changes in plasma membrane fluidity (Schuldes et al., 1998). Changes in the membrane lipid composition and order in resistant cells compared to sensitive ones are reported for a number of cell lines (Alone et al., 1991; Hendrich and Michalak, 2003). These findings suggest that membrane interactions can be involved in DOX effects in resistant tumor cells.

The antitumor drug thaliblastine (TBL) (Todorov, 1988) (Fig. 1) is shown to overcome MDR in DOX resistant P388/R-84 cells, and a direct binding to P-gp is reported as a possible mechanism of MDR modulation by this drug (Chen et al., 1993a). TBL exerts also high cytotoxicity in P-gp negative resistant cells and its effect could be further increased by hyperthermia (Chen et al., 1993b). Similarly to DOX, these observations point out to membrane interactions of TBL possibly involved in MDR cells.

In a number of studies we demonstrated that drug–membrane interactions play a role in modulation of MDR in tumor cells by catamphiphilic MDR modulators and found

* Corresponding author. Tel.: +359-2-979-3605; fax: +359-2-723-787.
*E-mail address:* pajeva@bio.bas.bg (I. Pajeva).
[1] Retired. Present address: Muhloch, 23845 Borstel, Germany.

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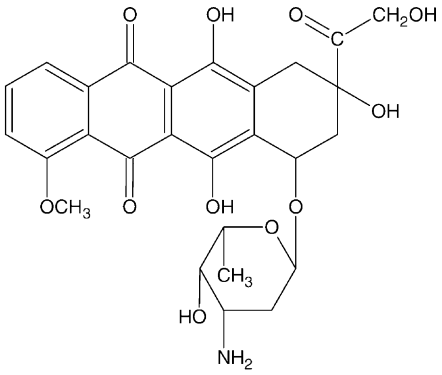
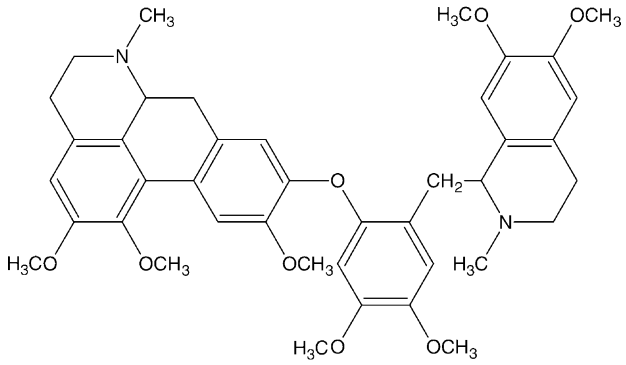



Doxorubicin




Thaliblastine

Fig. 1. Structures of the drugs studied.

a correlation between the membrane activity and anti-MDR activity of the studied drugs (Pajeva et al., 1995; Pajeva et al., 1996; Seydel et al., 1996; Pajeva and Wiese, 1997). Recent results of other research groups confirm this observation (Castaing et al., 2000, 2003; Hendrich and Michalak, 2003). It has been shown that stereo isomers of the same compound (e.g. *trans*- and *cis*-flupentixol) possess similar binding affinity to P-gp but different MDR reversing activity (Ford et al., 1990) and this difference can be related to differences in the strength of their membrane interactions (Seydel et al., 1994; Pajeva and Wiese, 1997; Wiese and Pajeva, 1997). These facts mean that the effects exerted on the membrane by MDR-related drugs should be considered. Therefore, we were interested to evaluate the membrane activity of the catamphiphiles DOX and TBL and to compare it to those of some well-known MDR modulators.

In this paper results on interactions of DOX and TBL with model membranes composed of phosphatidylcholine and phosphatidylserine are reported. Drug–membrane interactions were measured by differential scanning calorimetry (DSC) and nuclear magnetic resonance (NMR). The membrane activities of DOX and TBL were compared to those of the powerful MDR modulators *trans*-flupentixol and verapamil. The results suggest that drug–membrane interactions could play an essential role in DOX and TBL effects in resistant cells and that artificial membranes composed of neutral and negatively charged phospholipids could serve as an appropriate screening model for MDR modulation by catamphiphilic drugs.

## 2. Materials and methods

### 2.1. Drugs and chemicals

Doxorubicin hydrochloride, (±)-verapamil hydrochloride, 1,2-dihexadecanoyl-rac-glycero-3-phosphocholine (DL-α-phosphatidylcholine dipalmitoyl (DPPC)), 1,2-dihexadecanoyl-rac-glycero-3-phospho-L-serine (DL-α-phosphatidyl-L-α-serine dipalmi-toyl (DDPS)) and bovine brain phosphatidylseine (BBPS) (type III, fraction III) were purchased from Sigma Co., Germany. *trans*-Flupentixol was kindly supplied by H. Lundbeck (Copenhagen, Denmark). Thaliblastine hydrochloride was provided by Pharmachim (Bulgaria).

### 2.2. DSC measurements

All liposome suspensions were prepared in a phosphate buffer (pH = 7.4) by procedures that produce multilamellar vesicles (New, 1990). DPPC/drug mixtures were prepared by mixing appropriate amounts of DOX and TBL dissolved in methanol and DPPC dissolved in chloroform. At 30 °C the solvents were evaporated under argon and the samples were placed in a vacuum desiccator overnight at 4 °C. The phosphate buffer (0.0067 M) was added to the dried samples and the samples were incubated at 60 °C with vortexing for 2 h. In the DPPS experiments a stock solution of DPPS was prepared by dissolving DPPS in a phosphate buffer (0.067 M), warming to 64 °C with vortexing for 10 min and cooling to room temperature. DOX was solved in distilled water before adding to the appropriate amounts of the DPPS stock solution. DPPS/DOX, DPPS/TBL and DPPS/DOX/modifier mixtures were prepared by incubating the mixtures at 64 °C with vortexing for 40 min. In all preparations the incubation temperature was sufficiently higher than the main phase transition temperature, $T_{max}$, of DPPC or DPPS (about 42 and 54 °C, respectively). The vortex intensity used was 1200–1300 $min^{-1}$. The lipid concentration was 5 mg/ml in all samples and changes in pH were not observed. The experiments were done at different lipid:drug molar ratio in the interval from 1:0 (control) to 1:0.4. An ultra high sensitivity micro-DSC differential scanning micro-calorimeter (Setaram, France) with automatic data collection utility was used. A quantity of 200 μl of the liposome suspension and the same quantity of the reference (phosphate buffer) were heated at a rate of 0.5 °C/min and sensitivity of 50 mV. The temperature intervals vary from 0 to 70 °C. Every experiment was done at least twice and two runs were performed with some samples to make sure that the calorimetric response of the system was stable. The precision of

$T_{max}$ measurement was ±0.03 °C for DPPC and ±0.07 °C for DPPS. The type and strength of the drug–membrane interactions were evaluated on the basis of the following parameters: $T_{max}$, the temperature of the main gel-to-liquid crystalline phase transition of the phospholipid, defined as a peak (maximum excess specific heat) on the thermogram; $\Delta H$, the calorimetric enthalpy of the main transition, obtained by integration of the area under the transition curve and expressed in percentage of the area changing in relation to the area under the control endotherm (phospholipid only).

### 2.3. NMR measurements

BBPS was used for liposome preparation in NMR experiments. Samples containing 0.01 mg/ml $D_2O$ were sonicated using a Branson Sonifier B-12 (Branson Sonic Power Com., Damburg, Connecticut, USA) three times for 30 s at 40 W. This leads to a desired increase in temperature to 35 °C. After centrifugation the liposome preparation was allowed to equilibrate for 24 h at room temperature. This stock solution was diluted appropriately for the interaction studies. DOX and *trans*-flupentixol were solved in $D_2O$. The final pH was adjusted to pH = 5 so that the drugs were completely protonated. Three different concentrations of DOX were experimented: 1, 2 and 4 mM. Changes in pH were not observed. To 500 μl of the solution, liposomes were added in 5 μl portions. After the final portion of BBPS, a *trans*-flupentixol solution in $D_2O$ was added in a single portion of 40 μl (1 μM). Ethanol or DMSO was used as the standard to control field homogeneity. The experiments were performed with an AM 360L Spectrometer (Brucker, Darmstadt, Germany). Data acquisition included: 32 scans, 32 K FID, sweep width 4098 Hz, 0.25 Hz/Pt and homo nuclear presaturation to suppress the $H_2O$ signal. The obtained NMR spectra were characterized by the spin-spin relaxation rate $1/T_2$ expressed as the line width of the resonance signal (peak broadening in half peak heights). For calculations of peak-half widths a locally written curve-fitting program was applied. The significance in $1/T_2$ changes was confirmed by the observations as described in (Pajeva et al., 1996). The change in $1/T_2$ was related to a decrease in rotational freedom of the drug molecule in the presence of the phospholipid. The broadening was linearly dependent on lipid concentration within the range studied. The slope was used to determine the degree of interaction (Seydel, 1991).

## 3. Results

Fig. 2 shows the relative change in $\Delta H$ of DOX and TBL in up to 1:0.1 ratios. No significant changes in $\Delta H$ of the phase transitions of DPPC and DPPS were observed at DOX:lipid ratios up to 1:0.4. In contrast to DOX, still at lower concentrations, TBL showed decrease in the area under the main transition peak of DPPS indicating a specific interaction of the alkaloid with the negatively charged phosphatidylserine.

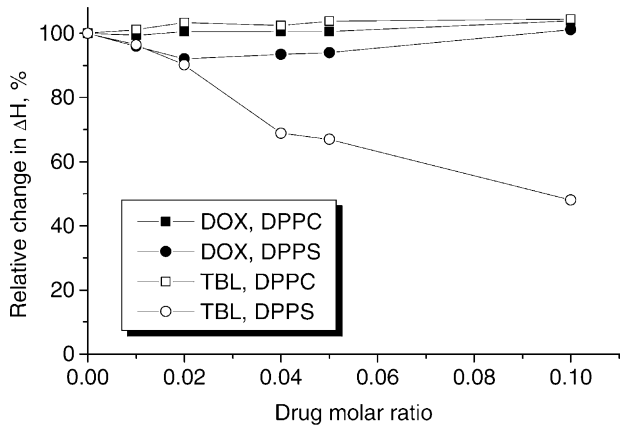



Fig. 2. Effects of increasing molar ratios of DOX and TBL on the relative change in the enthalpy $\Delta H$ of the main gel-to-liquid crystalline phase transition of DPPC and DPPS.

In Fig. 3, the relative change in $T_{max}$ of DPPC and DPPS upon increasing drug concentration is shown for DOX, TBL and verapamil. In the presented lipid:drug molar ratios all

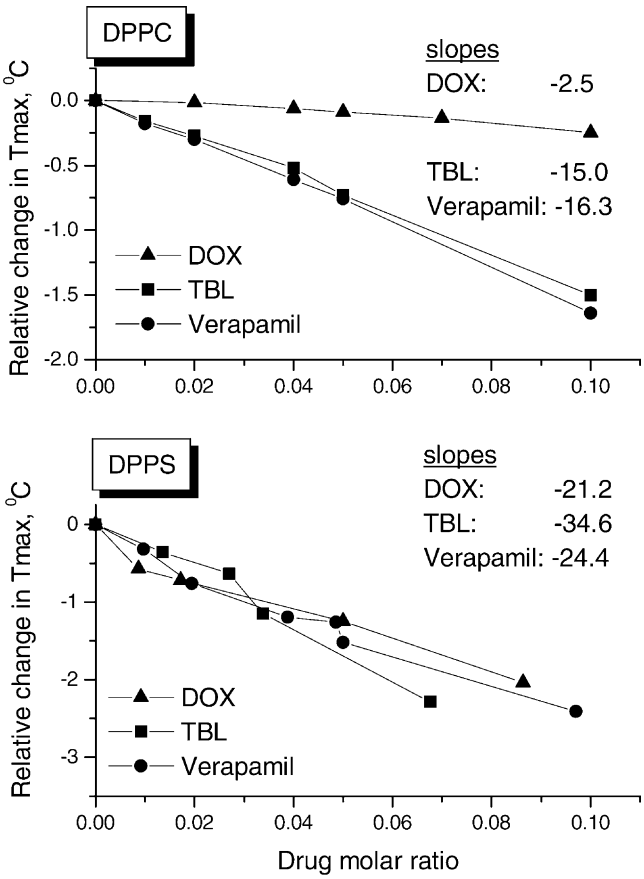



Fig. 3. Relative change in $T_{max}$ of DPPC and DPPS upon increasing drug content. The concentrations of the drug protonated forms are shown for DPPS that correspond to 86.4, 67.6 and 97.1% of protonation of DOX, TBL and verapamil, respectively, at experimental pH (7.4).

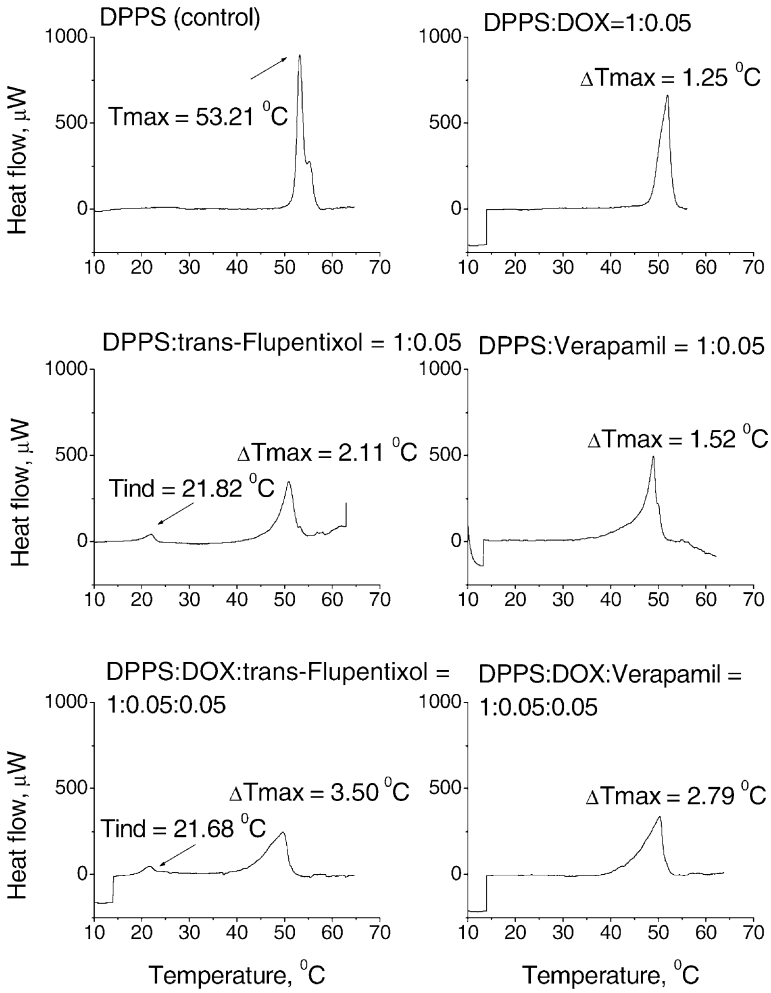



Fig. 4. Tracings of DPPS thermograms of DPPS alone (control) and upon addition of DOX, *trans*-flupentixol and verapamil alone and in combination at lipid:drug molar ratios as indicated on the themograms.

compounds cause decrease in $T_{max}$ that is much more profound for DPPS than for DPPC. The slopes of the linear fits show that the interactions of TBL and verapamil with the phospholipids are stronger than that of DOX.

Fig. 4 represents the DSC thermograms of DOX alone and at the MDR modulators *trans*-flupentixol and verapamil alone and in combination. As seen from the figure, at the same concentration of the drug protonated form, DOX shows the lowest decrease in $T_{max}$ (1.25 °C) compared to the control. *trans*-Flupentixol has the largest $\Delta T_{max}$ (2.11 °C) and verapamil is in the middle range (1.52 °C). Comparing the DPPS thermograms of DOX alone and the modulators alone to the thermograms of DOX plus the modulators at the same ratios, a further decrease in $T_{max}$ is observed that indicates a combined effect of DOX and the modulators on the phospholipid phase transition at the concentration studied. The observed $\Delta T_{max}$ shift can be interpreted as additive rather than cooperative one, considering the experimental error. As previously shown for a number of catamphiphilic MDR modulators (Pajeva et al., 1996) a new peak appeared on the thermogram of *trans*-flupentixol at a temperature $T_{ind}$ lower than $T_{max}$ (Fig. 4), indicating a specific interaction of this modulator with DPPS. $T_{ind}$ was concentration-independent and specific for a given drug. To check whether the appearance of new endothermic peak depended on the drug concentration, the lipid:drug molar ratios were further increased. No new peak was recorded for DOX up to 1:0.4 ratios. A new drug-induced peak, however, was registered for TBL (Fig. 5) corresponding to the observed decrease in the enthalpy $\Delta H$ of the main transition (Fig. 2). In Fig. 5, the DPPS thermograms of verapamil are also shown for comparison (Pajeva et al., 1996). Interestingly, both drugs demonstrated similar forms of the DSC thermograms indicating also similarity in their behavior in lipid environment. TBL again interacted stronger than verapamil inducing a new peak at lower temperature (33.65 °C) compared to verapamil ($T_{ind}$ = 32.80 °C) at the same drug ratios.

Fig. 6 represents a plot of increasing interaction of DOX ($1/T_2$ increase of the indicated spin system) with increasing BBPS concentration in the NMR experiments. The results of the NMR series obtained at 0.04 mM DOX concentration are shown. As seen from the figure the broadening of the

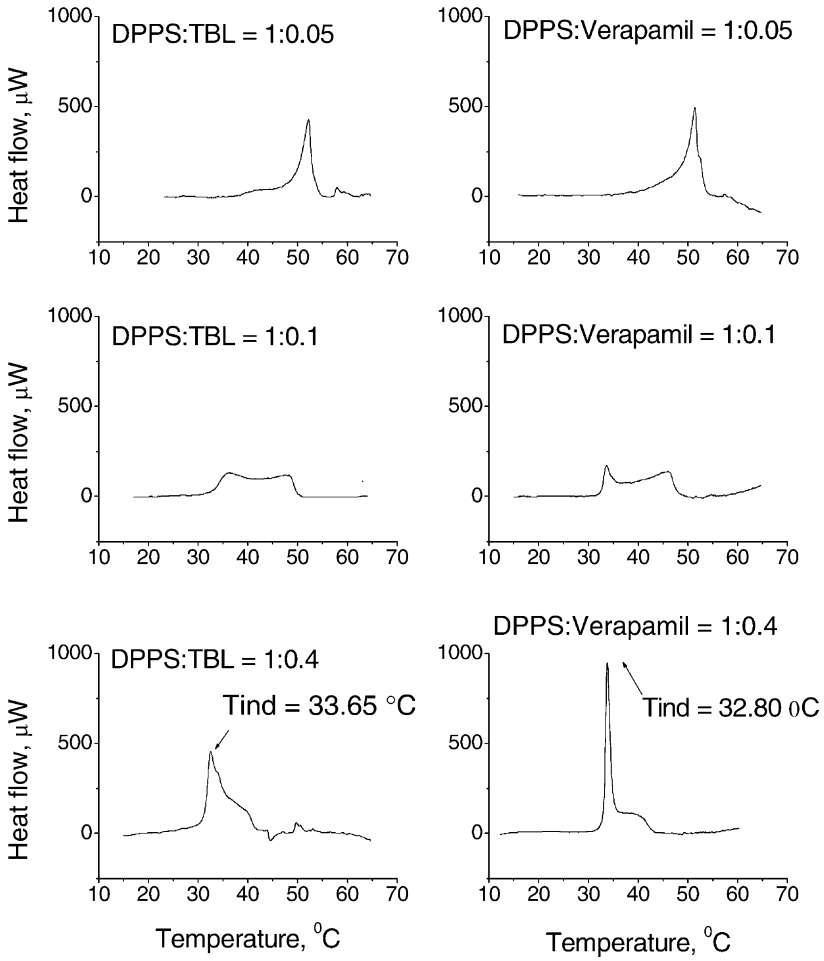



Fig. 5. Comparison of DPPS thermograms of TBL and verapamil at similar lipid:drug molar ratios as indicated on the themograms.

proton resonance signals are linearly dependent on the lipid concentration within the range studied. The slope of the proton signal of the $CH_3$ group is about 1.5 higher than that of the aromatic proton (although different in nature, the two spin systems can be compared as the relative change in $1/T_2$ is considered). The NMR experiments performed at lower DOX concentrations (1 and 2 mM) resulted in the same ratios of the slopes as observed at 4 mM DOX concentration

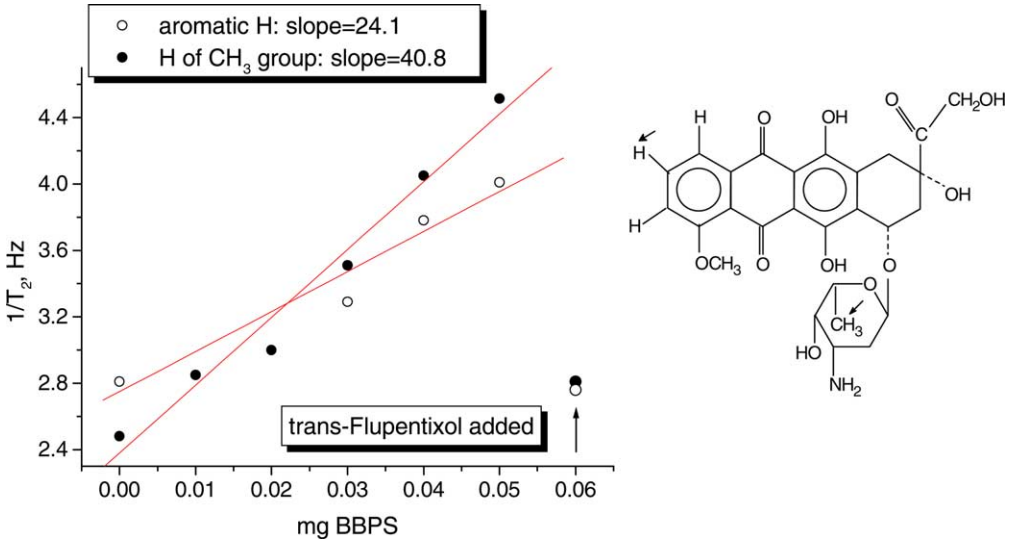



Fig. 6. Changes in DOX proton relaxation rate $1/T_2$ as function of increasing concentration of the phospholipid BBPS. The measured spin systems are indicated with arrows on the DOX structure.

(data not shown). The obtained slope of DOX $CH_3$–proton signal (40.8) is lower than those of the aliphatic protons of *trans*-flupentixol and verapamil, 96.2 and 42.8, respectively (Pajeva et al., 1996) at the same drug concentration. Although the direct comparison between DOX and these modifiers is not fully correct as the spin systems used are not identical, according to the slopes, DOX can be related to the drugs that exhibit strong interaction with the negatively charged phosphatidylserine (Pajeva et al., 1996). Addition of *trans*-flupentixol led to a complete reversal of the broadening of DOX resonance signals (Fig. 6) indicating that DOX binding to the phospholipid was replaced by binding of *trans*-flupentixol.

## 4. Discussion

The change in the enthalpy $\Delta H$ of the main gel-to liquid crystalline phase transition of DPPS indicates differences in the way the investigated drugs interact with the acidic phosphatidylserine at the concentrations studied (Fig. 2). The enthalpy decrease and appearance of a new peak on DPPS thermograms in case of TBL and the modifiers (Figs. 3 and 4) still at low lipid:drug ratios suggest a new phase transition probably related to a new domain formation between the drug and the phospholipid. This might be due to predominating electrostatic interactions of the positively charged drugs with the negatively charged phosphatidylserine at physiological pH. DOX has already been reported to form specific complexes with negatively charged phospholipids (Goormaghtigh et al., 1980; Triton and Yee, 1982; Nicloay et al., 1988; De Wolf et al., 1993; Speelmans et al., 1994). In our experiments, distinct from TBL and the modulators, DOX did not affect $\Delta H$ and did not induce a new peak on the DPPS endotherms suggesting no domain formation of DOX with DPPS at the studied ratios. The drug has previously been reported to interact with DPPC and the acidic phosphatidylglycerol without changing the enthalpy of the main transition (Constantinides et al., 1986). Our results confirm this observation but show stronger effect of DOX on $T_{max}$ decrease of DPPC compared to the results of Constantinides et al. (1986). At the same time, the observed $T_{max}$ dependence on DOX concentration was much more profound for DPPS than for DPPC (Fig. 2). Indeed buffers of different ionic strengths were used: 0.0067 M (0.0133 M total ionic concentration) in DPPC experiments and 0.067 M (0.133 M total ionic concentration) in DPPS ones (at 0.067 M DOX was not soluble in DPPC solution). However, no essential increase in the interactions of DOX with DPPC can be expected at higher ionic strength. Indeed, at the experimental pH (7.4) DOX is mainly protonated suggesting an active involvement of the charge interactions with the phospholipids. The higher ionic strength would favor the lipophilic interactions and, presumably, one can expect stronger interactions with DPPC. However, the same behavior of DPPC for different DOX concentrations in buffers with much larger difference in the ionic strengths (0.5 and 0.015 M total cation concentration) is reported (Constantinides et al., 1986) suggesting that the difference in the used ionic strengths cannot significantly influence the lipophilic interaction of DOX with DPPC. Thus, our result suggest that DOX interacts with both, neutral and acidic phospholipids, but affects more strongly the negatively charged ones and this can be related to the stronger electrostatic rather than lipophilic interactions. Also in the NMR experiments DOX exhibited a strong interaction with the negatively charged BBPS. Comparing the slopes of the aromatic and aliphatic proton signals (Fig. 6) one can conclude that DOX interacts stronger near the charged $NH_2$ group than near the aromatic part of the ring system. This may suggest that at phsyological pH, the positively charged amphiphilic DOX buries preferentially in the hydrophobic/hydrophilic interface of the acidic bilayer interacting primarily with the negatively charged head group of the phospholipid. Indeed, a self-association of DOX can be assumed, however, as reported in the literature (De Wolf et al., 1992), it can be observed at much higher DOX concentrations (2–17 mM) than those used in this study. According to the sharpness of the resonance signals obtained at lower DOX concentrations (1 mM) (data not shown) the self-association of DOX in the performed experiments is most unlikely. Even if partially so, the self-association should decrease upon addition of the phospholipid because of the increase in binding sites. As reported by De Wolf et al. (1992) the membrane-bound DOX has a lower tendency for self-association also because of the hydrophobic interactions of the drug with the lipids. Thus, the charge interactions between DOX and BBPS head group are more likely than the drug self-association. The evidence of head-group charge interactions is also supported by the recent findings that DOX is embedded within the membrane at the level of the polar head group and changes also the membrane conformation (Gallois et al., 1998).

In presence of DOX plus modifiers DPPS follows the same character of thermogram profile like in presence of the modifiers alone: decrease in $T_{max}$, broadening of the main peak and appearance of a drug-induced peak at constant $T_{ind}$ (Fig. 4). The presence of DOX and MDR modulators simultaneously at low concentration causes stronger decrease in $T_{max}$ and further broadening of the main peaks of the DPPS endotherms in comparison to DOX alone and modifiers alone at the same concentrations. This points to a combined effect of DOX and modifiers at low concentrations that can be related to further increase in the bilayer fluidity.

The results on TBL demonstrate that it is a membrane active drug. Its interaction with neutral phospholipds is stronger than that of DOX and comparable to that of verapamil. In contrast to DOX, TBL induces a new peak on DPPS thermogramsm, as the modulators do, that suggests ability of the drug to interact specifically with negatively charged phospholipids. This agrees with earlier data on high entrapment rate of TBL in acidic liposomes (Todorov and

Deliconstantinos, 1982). In general, the behavior of TBL in lipid environment reassembles that of the MDR modulator verapamil.

The evidence of strong interaction of DOX and TBL with negatively charged phosphatidylserine suggests a high concentration of the drugs in the membrane. As the phosphatidylserine molecule is very sensitive to surface charges and occupies preferentially the inner half of the membrane bilayer because of the smaller head group (New, 1990), a higher drug content can be expected in the cytoplasmic-faced leaflet. Thus, one can speculate that the strong DOX– and TBL–phosphatidylserine interaction could favor the trap of DOX and TBL at the inner leaflet of the membrane. Recently it has been proposed that the P-gp drug accesses its protein binding site from the inner leaflet of the lipid bilayer (Higgins et al., 1997, Rosenberg et al., 2003). One can speculate that being concentrated mainly in the cytoplasmic-faced part of the membrane, the drug might be easier "caught" and pumped out by the protein. Moreover, in the membrane the drug may adopt the "right" conformation that is necessary for its binding to the protein. The good correlation between the membrane activity and MDR reversing activity of some catamphiphilic drugs (Pajeva et al., 1996; Seydel et al., 1996; Pajeva and Wiese, 1997) confirms this possibility. Additionally, the interaction with phosphatidylserine may also influence the activation of the enzyme PKC that is mainly responsible for P-gp phosphorylation (Chaudhary and Roninson, 1992). In agreement, a correlation has been observed between PKC inhibition and membrane activity for some MDR modulators (Pajeva et al., 1996; Seydel, 2002).

Appearance of a drug-induced peak on DPPS thermograms is probably due to formation of drug-containing DPPS domains that coexist with drug-free domains thus leading to membrane heterogeneity. The role of this domain formation on P-gp functioning cannot be directly estimated from the presented results. However, an influence on the integral protein activity can be presumed as shown for a number of membrane integrated enzymes (Seydel, 2002).

Recently, more and more attention is paid to the role of the balance between the passive cellular influx and active efflux of the drug molecules for MDR reversal (Garnier-Suillerot et al., 2001; Hendrich and Michalak, 2003). It has also been demonstrated that P-gp active transport inhibition is unique to a given pair of a substrate and inhibitor (Barecki-Roach et al., 2003). The stronger interactions of verapamil and *trans*-flupentixol with phosphatidylserine than DOX, reported here, implies increase in membrane fluidity facilitating in this way the DOX passive diffusion through the membrane. It means also competition between the drug and modulators for interaction with the phospholipid. The replacement of DOX by *trans*-flupentixol as shown in the NMR experiments, suggests that the portion of the drug "caught" in the membrane by the acidic phospholipids can be released by a molecule that interacts more strongly with the phospholipid. In this way the drug can be set free to enter the cell and to reach the corresponding intracellular targets.

In summary, the presented results point to the role of the drug–membrane interactions for DOX and TBL effects in resistant tumor cells. They direct as well to the artificial membranes as a convenient screening model for potential MDR modulators.

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